THE MORPHOLOGICAL, HISTOCHEMICAL, AND BACTERIOLOGICAL EFFECTS OF IONIZING RADIATIONS ON THE GASTROINTESTINAL TRACT OF MICE

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The Morphological, Histochemical, and Bacteriological Effects of Ionizing Radiations on the Gastrointestinal Tract of Mice.

by

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DEDICATION:

For Carol

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For Carol

FOREWARD

Cosmic rays, medical x-rays, radioactive fallout, and nuclear reactor waste products expose modern man to considerable quantities of ionizing radiation (1). Concern for the biological effects of radiation has stimulated research in the medical and physical sciences. Studies of experimental animals, atomic bomb casualties, and victims of reactor accidents have shown that radiation can produce widespread tissue damage and death. In order to comprehend the mechanism of this tissue destruction, one must understand the intracellular effects of ionizing radiation.

Gamma and x-rays eject excited electrons from atoms within cells; these electrons lose energy by interacting with adjacent molecules to produce ionizations and free radicals. This has been called the "primary event." Although most of the energy absorbed by the irradiated cell is dissipated as heat, the energy stored in ions and free radicals may spawn further chemical or physical reactions. Energy may be transferred through a series of chemical events which irreversibly damage macromolecular bonds, or it may be dissipated as fluorescence, phosphorescence, and vibrational energy. The time from the initial wave of radiation until the physical or chemical event is measured in fractions of a microsecond; the development of detectable biochemical or physiologic

changes may take hours, days, or even longer (2).

The effect of ionizing radiation on the individual cell may take the form of: 1) chromosomal aberrations; 2) malignant transformation; 3) division delay; 4) mitotic inhibition, in which the cell undergoes no visible damage but ultimately degenerates owing to its inability to divide; and 5) interphase death, a rapid cellular degeneration seen in extremely radiosensitive cells or after extremely high doses of radiation. Malignant transformation follows subtle changes in the cell's genetic material that allow continued mitosis, but in an altered form. Chromosomal damage may take the form of single or double strand breaks; the type of damage appearing at the next metaphase will depend upon whether the cell was irradiated before DNA synthesis (when the chomosome responds as a single strand) or during DNA synthesis (when the chromosome reacts as a two-strand chromatid). Such damage, however, is not an important mechanism of cell death because the normal chromosomal structure is usually restored by a process involving oxidative metabolism and ATP formation (2).

The more extreme disruptions of cell division require an understanding of the cell's generative cycle. DNA synthesis (S) and mitosis (M) occur as separate, well-defined periods in the life cycle of mammalian cells.

The post-mitotic, pre-synthetic phase is known as G_1; the post-synthetic, pre-mitotic phase is known as G_2. G_1: S and G_2 comprise interphase. M and G_2 are the most radiosensitive stages of the cell life cycle; division is most easily delayed or blocked when the irradiated cell is in either of these stages and least readily delayed or blocked in G_1. The block in G_2 is reversible; irradiated cells pass into this stage but are delayed from going into mitosis. The duration of the delay depends on the radiation dose and on the life cycle stage of the cell at the time of irradiation. While cells are blocked in G_2, G_1 cells begin to catch up; the mitotic index, which falls after irradiation, may increase temporarily to values above normal ("mitotic overshoot") when cells that have accumulated in G_2 begin to divide semisynchronously (2,3,4). The phenomenon is common to all mammalian cells, although the dose relationships may vary.

A lethally-irradiated cell may undergo several normal division cycles and then degenerate during or after an abnormal mitosis; alternatively, the cell may attempt no further divisions but may enlarge to form a giant cell which eventually degenerates. The number of successful divisions for lethally irradiated cells varies inversely with radiation dosage (eg. five divisions for cells receiving 100 rads and one division for cells

receiving 1000 rads). An extreme example is the phenomenon known as "interphase death"; here, the radiosensitivity of the cell or the radiation dose is so great (10,000 rads or more) that nucleus and cytoplasm degenerate immediately without further cell division.

Radiation-induced cellular damage is not necessarily lethal; many cells absorb sublethal amounts of radiation, and their recovery processes repair the damage (5). These recovery processes involve passive, nonenzymatic, stereochemical reactions between DNA and other molecules. Oxidative phosphorylation, ATP formation, uninterrupted DNA synthesis, and de novo protein synthesis are not believed to be necessary for intracellular recovery (5). Thus, the extent of radiation damage is determined by the balance between macromolecular disruption and intracellular repair processes.

The search for "the lethal biochemical lesion" has been disappointing. A number of chemical alterations have been observed, but the majority appear to be a consequence rather than a cause of mitotic failure. Some evidence, however, does suggest that DNA synthesis is essential for cell survival. Radiation-induced inhibition of DNA synthesis has been demonstrated in cell cultures (6-8) and is an immediate effect of radiation rather than a consequence of transient mitotic delay, cell lethality, or shifting cell populations between different stages of the life cycle (5).

Degradation of existing DNA, as well as decreased synthesis, may play a role in mitotic failure. Cellular DNA may be denatured by the bombardment of ions and free radicals or destroyed by the activation of specific DNAases (2,5,9,10). A definite correlation between cellular DNA content and radiosensitivity has been established for a variety of bacterial, fungel, viral, and mammalian cells. Thus, DNA may well be the critical "target" which determines continued cell survival, function, and reproduction (5).

The sensitivity of individual cells to reproductive failure is roughly similar for all types of mammalian cells (2). Varying radiosensitivity among different tissues is due to the varying number of cells in each tissue, the varying rates of mitotic activity, and the varying number of viable cells necessary for function. Several organ systems are exquisitely radiosensitive and contribute to radiation sickness and death; the gastrointestinal tract plays such a prominent role that it was selected for study.

For three years, experiments were performed in the Gastrointestinal laboratory of the Yale University Department of Internal Medicine with the cooperation of the Department of Radiation Therapy, Division of Animal Care, Yale-New Haven Hospital Histopathology laboratory, and the Yale Computer Center. The results of these

experiments will be reported and discussed with reference to current theories of radiation sickness and death.

These studies could not have been performed without the help or guidance of several people. I wish to thank Miss Sylvia Diamond, Mrs. Trudy Schonberger, Mrs. Ruth Adams, Mrs. Sharayu Deshingkar, and Mr. Patrick Pepi for their technical assistance; Miss Helen Grate for her assistance with radiation procedures; Mr. Frederick Putt for his advice on histological procedures, Dr. Grace Wyshak and Mrs. Diana Fisher for their help with statistics and computer programming; Dr. Howard Spiro, Dr. Teodoro Herscovic, and Dr. Morton Kligerman for their constructive criticism; and especially Dr. Martin Floch for his guidance, patience, and encouragement during the past three years.

TABLE OF CONTENTS

INTRODUCTION

Following Hiroshima and Nagasaki, investigators realized that several organ systems contribute to the "post-radiation syndrome" and to radiation death. Atomic bomb victims demonstrated pathological signs and symptoms such as nausea, vomiting, diarrhea, malabsorption, gastrointestinal ulcerations, fever, dehydration, acidosis, leucopenia, purpura, infections, and hemorrhages (11-15). The manifestations of radiation exposure could be reproduced in experimental animals by whole-body X-irradiation, and they were subsequently divided into several dose-dependent syndromes (16-19).

If mice are given 50-100 rads, no perceivable illness occurs; however, longevity decreases (20), and the risk of malignancy increases. After 400 rads, a small percentage of mice will suffer an acute death approximately 11½ days later. Infection, hemorrhage, and anemia are prominent and are associated with widespread damage of lymphoid tissue and bone marrow. This is known as "typical radiation death", "bone marrow syndrome", "bone marrow death", or "hemopoietic death". The LD_{50} is 500-700 rads for these animals. As the radiation dose is increased, mean survival time (M.S.T.) remains near 11½ days, but the per cent of fatalities increases. As the percent of deaths approaches 100% (at 1000-1200 rads), mean survival time decreases

to 3½ days; the M.S.T. remains stable at this level until radiation doses of 15,000-20,000 rads are achieved. At LD_{100} doses, infection is uncommon, and gastrointestinal symptoms predominate. This syndrome is known as "acute intestinal radiation death, the "intestinal syndrome", or "gut death". At extremely high doses of total body radiation (20,000-40,000 rads) death may occur within a few hours. Shivering and convulsive movements (secondary to CNS damage) predominate, and this syndrome is known as "brain death".

Species, strain, age, and other host factors determine the radiation dosage and MST associated with each syndrome (18-21). The variation of survival time for individual mice may be three days more or less than the MST of the group; however, this variance is less at higher doses of radiation.

In addition to total body irradiation, exposure of any large portion of the small intestine to the appropriate dose of radiation will produce the acute intestinal radiation syndrome. The anatomical lesions of the gastrointestinal tract in this syndrome have been well-studied by light (22-31) and electron (32-37) microcopy.

The first changes after 1000 rads occur deep in the crypts of Lieberkuhn; at one hour after irradiation, nuclei and nucleoli are swollen. Chromatin material concentrates in central nuclear masses and in a thin rim

at the nuclear membranes. Mitotic figures disappear. Monomuclear infiltration occurs at four hours. During the first 24 hours, progressive vacuolation, pyknosis, karyorrhexis, and karyolysis take place. Disintegrating epithelial cells occupy positions close to the lumen of an intestinal gland, a site normally reserved for mitotic cells, and slough into the lumen of the gut.

On the second postirradiation day, the crypts begin to regenerate. Mitotic figures reappear at 24-48 hours after irradiation although some studies report recovery as early as four hours. A significant proportion of these new mitotic spindles demonstrate triphasic or other bizarre forms. Cellular destruction slows to control levels, pyknotic nuclei disappear from the crypts, cellular debris is cleared, and the crypts appear normal by 48-60 hours.

On the third postirradiation day, the villi appear grossly abnormal. Epithelial cells at the villus tip slough into the lumen of the gut and are not replaced for several hours. The earlier failure of mitosis in the crypts produces a temporary gap in the column of cells migrating up the side of the villus. At this time, most of the villi are short, stubby, and edematous; several are partially denuded. The epithelial cells remaining at the tip and along the upper third of the

villus are large, misshapen, riddled with vacuoles, and sometimes multinucleated.

By the fourth postirradiation day, the villi are reconstituted; only a few abnormal cells remain at the tip. By the sixth posirradiation day, the microscopic appearance of the intestinal mucosa has returned to normal.

Histological changes may be observed in other cells of the gut wall. Within a few hours after irradiation, goblet cells swell, discharge their contents, and cover the bowel lining with a thicker layer of mucus. Thereafter, the goblet cells increase in size and decrease in number; the mucus layer thins until villus recovery has been completed (28,29). Similar changes occur in gastric mucoid cells (33). Paneth cells are relatively radioresistant; only mild nuclear fragmentation and cytoplasmic degranulation take place following 1000 rads (26,28,36). These secretary cells, as well as gastric zymogen and parietal cells, demonstrate swelling and fragmentation of mitochondrial membranes or other subcellular membranes along with the disappearance of ribsomes from the endoplasmic reticulum (33,34,36). The intestinal lamina propria is infiltrated by neutrophils, small lymphocytes, and eosinophils on the first and second posirradiation day. On the second and third day, edema is prominent. The infiltrates and

edema resolve by the time that recovery of the villi has taken place (26).

PAS - Schiff preparations of the small bowel demonstrate decreased intensity of Feulgen-positive material within one to two hours after irradiation; tissue sections remain pale until 48 hours after irradiation (68). Biochemical studies of DNA synthesis in small bowel homogenates correlate well with these histochemical findings; DNA synthesis drops precipitously one hour after irradiation, is minimal at eight hours after irradiation, and recovers completely within 48 hours (69). Toluidine-blue sections demonstrate a parallel sequence of events for intestinal RNA content.

Histochemical studies of gastrointestinal enzyme activity in the irradiated animal offer conflicting results. In the normal small bowel, enzyme activity is intense in the supranuclear region of cells at the tips of the villi, moderate in the cells at the sides of the villi, and minimal in cells at the junction of the villi and the crypts (29). Outside the body, irradiation inactivates enzymes in aqueous solution. In vivo, irradiation increases enzyme activity, presumably through the rupture of subcellular membranes. Enzymes, which are normally separated from intracellular substrates, spread through the entire cell and catalyze destructive reactions (70). This enzyme release theory (of Bacq

and Alexander) has never been substantiated to date (29,30). Spiro and Pearse demonstrated increased duodenal cathepsin and non-specific esterase activity in the first forty-eight hours after irradiation; however, staining was weak and diffuse on the third postirradiation day. Recovery of enzyme activity began in cells at the sides of the villi on the fourth day and was completed by the sixth day (29). These authors showed that early increases of intracellular enzymes do not necessarily produce irreversible cellular destruction; however, the disruption of intestinal enzyme activity may contribute to the physiological disturbances observed in irradiated animals (29).

Radiation induces many functional disturbances of the gastrointestinal tract (38). Vomiting occurs in species where this reflex exists. Gastric secretion of pepsin and hydrochloric acid decreases (33,39). Pylorospasm and progressive gastric distention prolong gastric emptying time (40,41). The propulsive force of the intestine may be increased or decreased, depending on the relative excitability of cholinergic and adrenergic receptors in intestinal smooth muscle (38). Radiation-induced diarrhea may be reduced by diverting bile flow from the intestinal tract (42-44). Electrolyte concentrations and enzyme activity within the gut wall are

disturbed (45-49). Fecal weights increase to 2½ times normal values as intestinal contents are poorly absorbed (50,51,67). Intestinal uptake studies for sugars (52-55), electrolytes (56,57), amino acids (58), vitamins (57,59-62), fats (63), and drugs (64-66) show contradictory results but usually demonstrate malabsorption on or about the third day after irradiation (38).

Histological changes by themselves do not explain the decreased uptake of substances which are absorbed by different mechanisms. Alternative explanations of radiation-malabsorption include: 1) impaired pancreatic exocrine secretion, 2) altered intestinal flora, 3) shortened transit time resulting in inadequate exposure of intestinal contents to transport enzymes, and 4) deactivation of mucosal enzymes (50).

After a single LD_{100} dose of radiation, the general appearance of irradiated animals correlates well with the amount of damage to intestinal villi. The mice appear normal for 48 hours. On the third day, the fur is ruffled, the back is arched, and the animals have diarrhea (28). On the fourth day, symptoms are accelerated, and feces may be blood-tinged. Death occurs on the third to seventh day, but a small percentage of animals may survive the intestinal radiation syndrome to die a "hemopoietic death" seven to ten days later.

After a single LD_{50} dose of radiation, a less severe gastrointestinal reaction takes place. Survivors may live four to six weeks or longer; nonsurvivors usually

expire in the second postirradiation week with a syndrome characterized by bacteremia, hemorrhage, and anemia (18).

Postirradiation bacteremia has been observed for nearly fifty years, and the organisms involved are usually Gram negative bacilli (71-77). Earlier investigators histologically demonstrated bacteria in the lumen of the bowel adjacent to the denuded epithelial lining; consequently, they attributed Gram negative septicemia to cross-country bacterial invasion of the gut wall (71, 73,74,78,79). However, the clumps of bacteria were isolated and superficial, and these authors never satisfactorily demonstrated the progressive intestinal cellulitis which they postulated to exist. By taking daily blood cultures, Hammond demonstrated that endogenous postirradiation bacteremia does not occur on days 2-4 when damage to the intestinal mucosa is maximum; it appears on day 7-15 when the epithelium no longer shows histological damage (80). In a similar fashion, Shechmeister, Hammond and others demonstrated that artifical infection of irradiated animals, whether by oral inoculation, subcutaneous injection, or aerosol spray, produced the greatest incidence of bacteremia and death when bacteria were given on days 7-15 after irradiation (82-85). Death in this time period could not be produced by irradiating the abdomen alone, but

it did follow irradiation of the whole body with the abdomen shielded (17). Therefore, more recent investigators have related bacteremia and death in the second week after midlethal irradiation to factors other than the temporary denudation of the intestinal epithelium (16-18, 82-85).

Susceptibility of irradiated mice to infection, defined in terms of percentage deaths and rapidity of death after bacteremia, increases linearly until the fifteenth postirradiation day. At this time irradiated mice are five times more susceptible to infection than unirradiated controls; susceptibility then drops exponentially until control values are reached shortly after thirty days (83,84).

The post-bacteremic survival time of irradiated mice depends on the organism involved. Pseudomonas bacteremia is fatal in less than 24 hours; Proteus, two days; E. coli, three days; and Paracolon, four to five days (80). Even heat-killed, avirulent and attenuated virulent bacteria are lethal for irradiated mice during the second week after irradiation (82,86). In the same period, bacterial endotoxins enhance the lethal effects of radiation; however, results are contradictory and depend on the time of inoculation and the dose of radiation (87-90).

In the second week after midlethal irradiation, the mouse's defense mechanisms against bacteria and bacterial products are greatly impaired; indeed, several radiation induced abnormalities of the reticuloendothelial system have been described. The bone marrow, spleen, thymus, and lymph nodes are exquisitely radiosensitive and show extensive cellular destruction within hours after 400 rads (83,91); recovery, as judged by the weight of these organs, begins on or about the seventh day after irradiation. The migration of leucocytes, phagocytosis, antibody production, serum "Properdin" levels, and the bactericidal power of blood are markedly depressed by radiation (92-97).

In addition, the hemopoietic functions of marrow and lymphoid tissue are disturbed. Lymphocyte and neutrophil counts drop to minimal values on days 4-8 after irradiation; platelet counts, on days 9-10; and erythrocyte counts on days 8-12. By day fifteen, considerable recovery has taken place in both RBC and WBC counts (18,83).

As a complication of this pancytopenia, hemorrhage into vital organs or exsanguination often follow thrombocytopenia and at times may be the immediate cause of death. When bleeding is more subtle, anemia occurs three to six weeks after irradiation.

Many observers have noticed that irradiated animals ingest smaller quantities of food and water and lose weight in the second week after whole-body irradiation;

consequently, attempts have been made to implicate starvation and inanition in the etiology of death at this time. Indeed, the deprivation of food or the feeding of an inadequate diet to an irradiated animal may produce a number of metabolic disturbances, may alter the intestinal flora, and may enhance the animal's susceptibility to bacteria and bacterial toxins (98-105). On the other hand, nutritional deficiencies alone fail to abolish the bactericidal power of the blood, liver, and spleen in the manner that radiation alone does (104). Furthermore, the inoculation of 0.1 microgram or less of Gram negative endotoxin by itself may bring about a reduction in oral intake and body weight (106,107). In this manner, food intake influences endotoxin tolerance, but the presence of endotoxin regulates food intake. Therefore, depressed oral intake may contribute to the debility observed in the second week after irradiation, but it is not the major mechanism of death (108,109).

Although the signs and symptoms of the various post-irradiation syndromes have been well described, therapeutic attempts have been disappointing. A number of potentially protective agents have been studied, and a few (eg. substances which induce hypoxia, such as histamine, cyanides, catecholamines, para-amino-propiophenone, some anesthetics, or tryptamine; thiols

and disulfide compounds such as mercaptoethylamine, glutathione, and 2-mercaptoethylguanidine; spleen or bone marrow homogenates; nucleoprotein preparations; and antibiotics) have achieved limited successes under special circumstances (5). A review of each compound and the specific instances in which it is effective is beyond the scope of this dissertation; however, two classes of radioprotective compounds were chosen for study and deserve further mention.

Following earlier observations that spleen-shielding may protect irradiated animals, Lorenz and others demonstrated that injections of spleen or bone marrow homogenates could prolong life, modify radiation injury, and enhance resistance to infection in irradiated mice (110-114). The same effects were also achieved by injecting; 1) homogenates of infant spleens into irradiated adult mice; 2) marrow homogenates from heterologous species into irradiated mice, and 3) uncontaminated nuclear fractions of spleen cells into irradiated mice. Cytoplasmic subcellular fractions were inactive, and nuclear fractions pre-treated with Deoxyribonuclease or trypsin (but not Ribonuclease) lost their protective ability (115-120). Accordingly, these investigators concluded that the active component of marrow and spleen homogenates was a deoxyribonucleoprotein (119,120).

Indeed, DNA injections prolong survival in irradiated rats; in each instance, the duration of survival and the percentage of survivors depend on the amount and molecular weight of the DNA preparation, not on the homology of donor and recipient species (121,122). DNA labelled with tritiated thymidine, after intraperitoneal injection into mice, localizes in the spleen, lymph nodes, bone marrow, free lymphocytes, and in the crypts of the small intestine (122,123). Presumably, radiation increases the permeability of these cells to the entrance of macromolecules, including DNA; and this pre-formed DNA or its breakdown products can be utilized for the repair of radiation damage (122,124-127).

In irradiated animals, antibiotic therapy produces variable and frequently disappointing results. When irradiated mice are inoculated with exogenous bacteria and with antibiotic preparations (eg. streptomycin, terramycin, aureomycin, chloromycetin, or penicillin, the incidence of bacteremia is reduced; in some cases, mean survival time is prolonged but without any increase in the ultimate percentage of animals surviving (127-129). However, such findings are not consistently reproducible because the efficacy of antibiotics is determined by many variables.

One determinant of antibiotic efficacy is the dose of radiation. Antibiotic treatment has a protective effect

only after moderate exposure to radiation (less than 700 rads); after higher doses, mice die before septicemia takes place (130). Consequently, antibiotic therapy is relatively useless in the acute intestinal radiation syndrome compared to its protective effect during the bone marrow syndrome (17,18).

Because the virulence of different experimental bacterial inoculations varies widely, the route of injection, the dosage, and the bacterial species employed will greatly influence the outcome of such studies. Similarly, the type of antibiotic, the dosage, the timing of injection, and the route of injection are also important. Parenteral antibiotics reduce the incidence of bacteremia but do not prolong life significantly; oral preparations, and especially those which are poorly absorbed across the intestinal mucosa, have been shown to prevent bacteremia, prolong life, and in some instances, prevent death (131). When antibiotics are given prior to or immediately after irradiation, they must be continued for at least three weeks in order to obtain maximum effect. The shorter the period of treatment, the greater will be the postirradiation mortality (131).

Postirradiation survival has been related to the suppression of intestinal coliform bacteria; when antibiotics fail to render the stool coliform-free, they have no effect on duration or rate of survival (131). Conversely, the incidence of diarrhea, melena, and weight

loss is significantly reduced in germfree or coliform-free mice, and such animals routinely survive radiation doses which are lethal for conventional mice (19, 131-133).

With the improvement or experimental design and bacteriological techniques, some of the earlier conclusions must be re-examined. When previous investigators inoculated irradiated animals with exogenous bacteria and noted the subsequent appearance of these organisms in the host animal's blood (82-85,98,103,104, 128,134,135), they actually learned very little about the host animal's susceptibility to its own gastrointestinal flora. When these same investigators demonstrated Gram negative bacteria in the blood of irradiated animals (80-82,101,104,127), they interchanged the terms "bacteremia", "septicemia", "sepsis", "infection", and invasion" freely and perhaps improperly. The presence of bacteria at death did not necessarily mean that infection contributed to the animal's demise, and, conversely, the absence of bacteremia did not prove that infection contributed nothing (86, 127).

The early observers vigorously sought evidence to prove that progressive intestinal cellulitis was the source of pre-terminal bacteremia. Many were able to demonstrate increased bacterial counts in: homogenates of liver, spleen, mesenteric lymph nodes, kidney, and bone marrow (81,82,101,103); homogenates of isolated

bowel segements containing stool (85); stool speciments excised from the intestine (105); mixtures of intestinal tissue and stool (135); and defecated stool specimens (136).

Upon more careful analysis, it is obvious that bacterial counts from such specimens do not necessarily support the intended conclusion. Small numbers of intestinal bacteria normally find their way into mesenteric lymph nodes, the liver, spleen, and kidney. Following irradiation, the proliferation of bacteria in these organs prior to bacteremia may reflect the reticuloendothelial incompetence of these organs rather than invasion of the gut wall. Furthermore, bacterial proliferation in feces is not necessarily evidence for proliferation in the bowel wall. The contamination of intestinal homogenates with gross quantities of fecal material produces bacterial counts which reflect growth in the stool rather than in the intestine itself. Colon feces and defecated stool samples contain material which has travelled the entire length of the alimentary tract. Bacterial counts from such specimens represent a composite of oral, gastric, intestinal, and colonic bacteria and may obscure local variations in bacterial populations.

Despite inferences to the contrary, bacterial invasion of the gut wall has never been demonstrated satisfactorily by bacteriological techniques. In the

1950's, *in vitro* culture methods were inferior to present methods. Media consisted of plain agar, blood agar, or broth; incubation was primarily a matter of keeping the cultures warm (18); and recovery of intestinal bacteria was poor by present day standards. To investigators who used such techniques, lactobacilli appeared irregularly throughout the digestive tract, the stomach was sterile because of its low pH, the large bowel contained coliforms as the predominant organism, and the small bowel was not very important.

In 1965, Schaedler and Dubos demonstrated a reliable method of anerobic incubation which enabled them to harvest a greater number and variety of intestinal bacteria than their predecessors (137). They selectively incorporated stimulants and inhibitory substances into their media and recovered fastidious and nutritionally more demanding species (eg. clostridia, bacteroides). These authors also developed techniques to differentiate bacteria living freely in the lumen of the gut from those adhering loosely to the mucus covering the epithelium and from those that were buried beneath the surface of the gut wall (138). Schaedler and Dubos used these techniques to study the development of the mouse gastrointestinal flora from birth to adulthood, and, by paying careful attention to quantitative procedures, achieved

greater precision and reliability in bacterial counts than previous investigators (137,138). Accordingly, with such improved techniques now available, we decided to re-evaluate the activity of gastrointestinal bacteria in the irradiated animals.

Three major experiments were performed. Experiment I was a histological study designed: 1) to describe the microscopic anatomy, mitotic activity, nucleic acid activity, and enzyme activity in the intestine of irradiated mice; and 2) to test the ability of exogenous intraperitoneal DNA to modify these parameters of radiation injury.

Experiment II was a bacteriologic study designed: 1) to describe the normal gastrointestinal flora for weanling white mice of a particular strain; 2) to test the effect of irradiation on bacteria; and 3) to demonstrate bacterial invasion of the gut wall by culture or by tissue Gram stain techniques.

Experiment III was a bacteriological study designed: 1) to describe the normal colonic flora for adult white mice of the same strain; 2) to test the effect of radiation on these bacteria; 3) to demonstrate bacterial invasion of the colon wall by culture or by tissue Gram stain techniques; 4) to test the effect of antibiotics on the colonic flora in normal and irradiated mice; and 5) to study survival in antibiotic-fed and conventional irradiated mice.

MATERIALS AND METHODS

Experiment I

Forty two male and female white mice of the Charles River strain were employed in this experiment. Twenty four were given 900 rads (250 kv., 117 rads/min., filtered by a $\frac{1}{2}$ aluminum-$\frac{1}{2}$ copper filter, HVL-1.65 mm. copper, for 7.69 min., in a 10 section circular mouse container mounted on a turntable with the target 54 cm. from the cone) of whole-body x-irradiation. Immediately following irradiation, twelve animals were given an intraperitoneal injection of 300 micrograms of DNA ("Highly Polymerized DNA" from Nutritional Biochemical Corp., Cleveland) which had been suspended at 4^{0}C in 0.14M saline for 24 hours (122). Twelve controls received an injection of 0.14M saline. At 1,2,4,8,24 and 48 hours after irradiation, pairs of DNA-injected and saline-injected mice were killed by a quick blow to the head. As further controls, two mice were given saline and killed immediately without treatment; two mice were given saline and killed immediately; two mice were given DNA and killed immediately; six mice were given DNA and killed at 1,2,4,8,24, and 48 hours after injection; and 6 were given DNA before irradiation and were killed at 1,2,4,8,24, and 48 hours after irradiation.

From each animal, portions of mid-jejunum, mid-ileum, and mid-colon were excised and quick-frozen in

liquid nitrogen or fixed in Lillie's formalin. Formalin-fixed tissues were embedded in paraffin, and sections out at 5 micra were stained with hematoxylin and eosin, Schiff's reagent and Light Green to demonstrate DNA, and Methyl Green-Pyronin to demonstrate DNA and RNA (139, 140). Quick-frozen tissues were mounted on liver slices for support and stored in plastic bags at -70°C. Cryostat sections, cut at 4 micra, were stained with indoxyl acetate to demonstrate cathepsin and non-specific esterases (139). Enzyme activity in the epithelial cells of the villus tip, villus side, villus base, and crypt gland was evaluated by a histochemistry technician unfamiliar with the treatment given to each animal. This activity was determined by the intensity of the histochemical stain which was graded from 0 to 4. Four micra sections of liver from each animal served as controls for the staining procedure.

The Feulgen-stained sections were used for mitotic counts. One thousand consecutive crypt epithelial cells from the basal regions of consecutively scanned crypt glands were counted. The mitotic index was expressed as the number of mitotic figures per 100 crypt cells (141,142).

Experiment II

Twenty eight white male weanling (3 week old) mice of the ICR strain were employed in this experiment.

Fourteen were given 1200 rads (114 rads/min. for 10.53 min. with the previously described irradiation equipment) of whole-body x-irradiation. Fourteen served as unirradiated controls. At 1 hour, 1 day, 2, 3, 5, 7, and 10 days after irradiation, two mice from each group were killed by a quick blow to the head. The fur was sprayed with a germicidal aerosol (Staphene Spray, Vestal Laboratories, St. Louis), and the abdomen was entered by sterile dissection. From this point in the experiment, all specimens for bacteriologic assay were handled with aseptic technique. The entire stomach as well as 2 cm. segments of mid-jejunum and mid-colon were excised and placed individually in sterile petri dishes. For histochemical purposes, a specimen of each organ was mounted on a slice of liver, quick-frozen in liquid nitrogen, and stored in a plastic bag at -70°C; at a later time, 4 micra cryostat sections were stained with the Brown-Brenn technique for bacteria (143) and studied by light microscopy.

Many of the following bacteriological techniques have been described previously (137,138,144). From the remainder of each organ, 0.1 gram of lumenal contents was removed and cultured within 15-30 minutes. A flamed No. 2 bone curette (Sklar Instruments) was found to be reliable for measuring this amount. The 0.1 gram of specimen to be cultured was diluted with 9.9 ml. of sterile norite A charcoal water; charcoal water was

prepared by filtering distilled water over norite A (Nutritional Biochemicals Co., Cleveland). This first tube containing a 1:100 (10^{-2}) dilution was placed on a vigorous shaker (General Purpose Variable Speed Eberbach Shaker) for five minutes to evenly disperse the particles of organic material. The resultant emulsion was then serially diluted by adding 1 ml. from the first tube to 9 ml. of charcoal water, and so on, until five tubes were prepared. The first tube prior to serial dilution was a 1:100 dilution, and the last tube represented a 1:1,000,000 (10^{-6}) dilution.

A loopful from each tube selected for analysis was then streaked on the appropriate solid bacteriological media. The 4mm loop delivered .01 ml. of fluid; therefore, growth at the streak taken from the first tube represented a $10^{-4}(10^{-2} \times 10^{-2})$ dilution of the total number of colonies, and growth from the last tube represented a $10^{-8}(10^{-6} \times 10^{-2})$ dilution. The first, third, and fifth tubes were selected for streaking, and final counts were respectively 10^{-3}, 10^{-5}, and 10^{-7} dilutions of the original specimen. If 3 colonies were found on a plate representing a 10^{-5} dilution, the bacterial count for this specimen would be recorded as 3×10^{5} organisms per gram of stool. When bacterial counts increased, higher dilutions were used so that the number of colonies per petri dish never exceeded 200.

At the same time, approximately 0.1 gram of each organ was sliced open longitudinally, placed in a tube of sterile norite A charcoal water, and shaken vigorously for five minutes. From previous experiments, we had determined that one washing with five minutes of shaking was sufficient to lift away the mucus overlying the intestine without damaging the tissue itself. The washed specimen was removed and weighed to three decimal places on a semi-automatic balance (Sartorius-Werke, Gottingen). The wash water was then serially diluted and cultured on the appropriate media.

The washed specimen was diluted to 10cc. with sterile norite A water and homogenized by a motorized teflon pestle in a grinding tube (TRI-R STIR-R, Model S63C, TRI-R Instrument Co.). Serial dilutions of this homogenate were streaked on the appropriate media.

Five selective media were employed for each specimen; they have been described previously (137). Medium A was employed for total counts, and two plates were streaked; one was incubated aerobically and the other anerobically. Medium C was used for the isolation of Bacteriodes species and Clostridia species, medium G for Lactobacilli, and Enteroccoccus M medium for Gram-positive cocci; each of these was incubated anerobically. Medium E was used to isolate coliform organisms and was incubated aerobically.

A standard incubator (Precision Scientific Co.) was employed for aerobic incubation; aerobic organisms were incubated for twenty four hours. For obligate anerobes, we used an anerobic incubator (National Instrument Co.) which was maintained at anerobic conditions by bringing it to a negative pressure of 21 inches of mercury with a standard vacuum pump and then flushing it five times with equal parts of carbon dioxide and nitrogen. An iron pad soaked in copper sulfate solution served as an oxygen scavenger within the incubator. The incubator was maintained at a negative pressure of nine inches of mercury and at a temperature of 37° for 48 hours.

Colony counts for total aerobic and total anerobic growth were made from medium A. These were correlated with counts from the other, more selective media. For this initial experiment, bacterial identification was accomplished primarily by comparing colony morphology on each medium with that of known specimens on similar media in our laboratory. Gram stains, blood agar subcultures, and sugar slants were employed on a spot-check basis to assess the accuracy of our identifications.

Bacterial counts (bacteria/gram of feces or tissue homogenate or bacteria/cc. wash water) were determined

for each specimen and converted to their logarithmic equivalent. The logarithm of each bacterial count was key-punched onto a separate computer data card along with such information as the presence or absence of irradiation, the time of sacrifice and the type of specimen (eg. stomach contents, jejunal wash, colon homogenate, etc.). When no bacteria were found on a given plate, it was necessary to record a small constant (10 or 10^1) to avoid the imaginary expression log 0. A programmed factorial analysis of variance was performed on an IBM 7094 computer; and mean bacterial counts, marginal means, F ratios, and significance levels were calculated.

Throughout the course of the experiment, the mice were housed ten per cage in plastic wiretop cages with sawdust litter. There were two pint-sized water bottles and one feeding bin per cage. The diet consisted of unrestricted quantities of water and Purina Laboratory Chow. The cages were cleaned and the water bottles were changed every three to four days. No attempts were made to prevent fighting, cannibalism, or coprophagy.

Experiment III

One hundred adult (90 day old) white mice of the ICR strain were employed, 40 in the initial phase of the experiment. Ten were untreated and served as controls. Ten were given 1200 rads (with the techniques mentioned

above) of whole-body x-irradiation. Ten were started on oral antibiotics and then given 1200 rads; and ten were given antibiotics only. Two mice from each group were sacrificed one day, three days, five days, seven days, and ten days after the day of irradiation. With the sterile techniques described in the previous experiment, the abdomen was entered, a segment of mid-colon was saved for histological purposes (as described in Experiment II), and 0.1 gram of colonic feces was serially diluted and cultured.

Incubation proceeded as described previously, but greater attention was paid to bacterial identification. Gram stains were made from each type of colony that could be harvested from the C,E,G, and Enterococcal media. From the C media, Clostridia were identified as Gram positive rods with or without spores; whenever present, their growth was confirmed on anerobic, blood agar subculture. The Bacteroides species were thin, pleomorphic, fusiform, or small Gram negative rods which grew only anerobically on blood agar (as small, clear or gray colonies). Gram stains from the G medium revealed the large, pleomorphic, or thick short Gram positive lactobacilli. As these colonies aged, they lost their ability to retain the crystal violet stain; they could be seen as Gram positive, mottled with patches of Gram negative staining, or entirely as Gram negative organisms. In these instances, characteristic morphology

appeared after blood agar subculture. Colonies from the Enterococcus-M medium were stained to identify Lactobacilli, Streptococci, and the larger Gram positive cocci of the Micrococcus species. When identification was uncertain, colonies were transferred to blood agar and incubated anerobically and aerobically. In the antibiotic-fed animals, Gram staining revealed both Gram positive and Gram negative yeast forms and hyphae; no attempt was made to identify fungi more precisely.

Data was recorded in the sam manner as above, and a factorial analysis of variance was performed on the 7094 computer. Mean bacterial counts, marginal means, F ratios, and, significance levels were calculated. The tissue sections were stained by the Brown-Brenn technique for bacteria in tissues (140) and were studied by light microscopy.

Throughout the course of this experiment, mice were housed ten per cage in plastic wiretop cages with sawdust litter. There were two pint-sized water bottles and one feeding bin per cage; the diet consisted of unrestricted quantities of water and Purina Laboratory Chow. For the antibiotic-fed animals, Neomycin Sulfate (Mycifradin Sulfate sterile powder, Upjohn) at a concentration of 10 grams/liter and Potassium Penicillin G (Squibb) at a concentration of 4 million units/liter were dissolved in the drinking water. Antibiotics were

started four days prior to irradiation and were maintained throughout the course of the experiment. At three to four day intervals, the antibiotic solutions in the drinking bottles were changed to maintain antibiotic activity. For all animals, the cages were cleaned at three to four day intervals; again, no attempt was made to prevent fighting, cannibalism, or coprophagy.

Sixty male mice of the same age and strain were employed in a survival study. Ten were untreated, twenty were given 1200 rads of total body radiation, twenty were irradiated and given antibiotics, and ten were given antibiotics only. The mice were housed ten per cage, fed, and given drugs as described above. At eight hour intervals for thirty days, the cages were checked and dead animals were removed. The time of death for each mouse was recorded as well as the daily count of living and dead animals.

RESULTS

Experiment I

In the small bowel, Morphologic changes include mononuclear infiltration of the crypts at one hour, pyknotic or abnormally vesicular epithelial nuclei in the crypts at two hours, and inclusion bodies and debris that stain heavily for DNA at four hours (see Fig. 1). These changes increase up to 24 hours and partially subside at 48 hours. In the colon, morphologic changes are more subtle; mononuclear infiltration of the glandular epithelium and lamina propria appears at 2-8 hours, and nuclear inclusions appear in these regions at 8-24 hours (See Fig. 2). No such changes were observed in unirradiated animals.

Most striking was the immediate disappearance of mitotic figures from the jejunal and ileal crypt epithelium of all irradiated animals (See Fig. 3). The mitotic index dropped precipitously within one hour after irradiation, began to recover at 24 hours, and recovered completely by 48 hours. There were no significant differences in the mitotic indices of mice receiving saline after irradiation, DNA after irradiation, or DNA before irradiation. Unirradiated animals maintained a constant mitotic index of 3.0% except for a spurt of mitotic activity at two hours after the DNA injection.

In unirradiated animals, crypt epithelial cells stained more intensely for DNA and RNA than villous epithelial cells. Jejunal cells stained more intensely than ileal or colonic glandular cells. In the jejunum of some irradiated animals, crypt epithelial cells stained as lightly for RNA as cells in the villi, implying some loss of crypt RNA content.

In unirradiated mice, Cathepsin (See Tables 1a-1j) and Nonspecific Esterase (See Tables 2a-2j) activity was greatest at the villous tips, decreasing progressively down the sides of the villi toward the junction with the crypts. These enzymes appeared predominently in the supranuclear region of epithelial cells. No sustained changes in the content or distribution of cathepsin and non-specific esterases could be seen in the first 48 hours after irradiation, and no effect of DNA could be found.

Experiment II

In unirradiated weanling mice, gastrointestinal bacteria live in distinct, well-localized populations rather than in a random mixture. The stomach (See Tables 3a-3c) contained large numbers (10^7-10^8) of anerobic lactobacilli in close relationship to the gastric wall: these organisms were recovered in large numbers (10^7-10^8) from gastric washes and homogenates as well as from gastric contents. In contrast, the jejunum (See Tables 3d-3f) contained large numbers (10^7-10^8) of anerobic lactobacilli in the lumenal contents but only a moderate

number (10^5-10^6) in the washes and homogenates.

In the unirradiated mouse of this age, the colon contained bacterial populations which were in a state of flux (See Tables 3g-3j). The most stable organism at this time was the anerobic lactobacillus, which was recovered from the stool (10^8-10^9), wash (10^6-10^7) and homogenate (10^5-10^6). The colonic feces contained a large number of aerobes (10^8) early in the course of the experiment, but these bacteria decreased to 10^5 near the end. A similar decrease was observed in the colon wash, and practically no aerobes were recovered from the homogenate at any time. Coliforms did not appear in the stool until the fifth sampling period, when they were recovered at 10^7 organisms/gram feces; their numbers declined to 10^5 by the end of the experiment. Except for one occasion, no coliforms were recovered from colon washes or colon homogenates. The number of Bacteroides in colonic feces was moderate (10^6-10^7) but highly variable. They were recovered at 10^3-10^5 in the colon wash and only on sporadic occasions in the colon homogenate. At no time were Clostridia recovered on the C medium nor anerobic streptococci on the Enterococcus M medium.

In the colonic feces (See Tables 3g,4, and 5) of irradiated weanling mice, there was a significant (p less than .01) increase in total aerobes on the fifth (10^7 versus 10^5 for controls, seventh (10^9 versus 10^5

for controls), and tenth (10^8 versus 10^6 for controls) days after irradiation. In addition, coliforms were significantly (p less than .01) increased, especially on the seventh (10^8 versus 10^4 for controls) day after irradiation; however, the recovery of coliform bacilli was erratic, and this increase did not persist to the end of the experiment. In a similar fashion, there were several other statistically significant differences between irradiated and control mice; however, as will be discussed below, such differences were within the variability of existing bacteriological techniques. There were no significant increases in bacterial counts for the colonic wash water (See Table 3h) or colonic homogenates (See Table 3j). Therefore, although some bacteria proliferated in the colonic stool of irradiated mice, these organisms did not invade the mucus layer nor the colonic wall itself.

On microscopic examination, the mouse stomach (See Fig. 4) was composed of glandular and nonglandular portions; the non-glandular areas were lined with stratified squamous epithlium. Gram positive rods and cocci, presumably lactobacilli, were seen in the lumen of the stomach and in the mucus layer overlying both glandular and non-glandular areas. In the jejunum (See Fig. 5), lactobacilli were confined to the lumenal debris. In the lumen of the colon small Gram negative rods, larger Gram-negative fusiforms, and Gram positive

rods could be seen. In both the small and large bowel, bacteria were sometimes seen in the mucus layer covering the epithelium and, on rare occasions, just beneath the surface of the epithelium. In the latter situation, bacteria were not clearly in the same focal plane as the tissue and appeared to overlie the specimen. No micro-abscesses and no evidence of bacterial invasion could be seen histologically.

Two of the mice died before they were cultured. These deaths occurred suddenly on the eighth and ninth days after irradiation. In a period of 4-6 hours, pallor and prostration terminated in death. Upon gross examination of organs, black stook was found in the intestine, and small clots or punctate hemorrhages were noted in the stomach and small bowel.

Because the gastrointestinal flora of the weanling mouse is not firmly established, we were not certain whether the radiation-induced bacterial changes seen in these mice could be reproduced in adult animals. Accordingly, we undertook a study of the colonic fecal flora in irradiated adult mice.

Experiment III

In the colonic feces of unirradiated adult mice (See Table 6a), anerobic bacteria (10^7-10^8) greatly outnumbered aerobes (10^6). The most numberous organism was the anerobic lactobacillus (10^8). Bac-

teroides were present in moderate numbers (10^6-10^7), and coliforms in comparatively small numbers (10^5).

In the colonic feces of irradiated adult mice (See Tables 6a,7,8), there is a significant (p less than .01) increase of coliforms (up to 10^{10} versus 10^5 for controls) and total aerobes (up to 10^{10} versus 10^7 for controls) on the tenth day after irradiation; this agrees with the findings in weanling mice. In addition, there is an equally significant (p less than .01) but less impressive increase in total anerobes on the seventh (10^9 versus $10^{7.5}$ for controls) and tenth (10^{10} versus 10^8 for controls) days after irradiation; this finding is not observed in weanling mice.

In the antibiotic-fed animals, no bacteria were recovered from the time of the first culture to the end of the experiment. Anerobic and aerobic fungi were the only organisms recovered on all five media (See Table 6b), and no significant increases (See Tables 7 and 8) were found in irradiated animals. No fungi were recovered from animals which had not received antibiotics.

On microscopic examination of the colon, the mucosal surface appeared unbroken for all animals. Fusiforms, small Gram negative bacilli, and Gram positive rods and cocci were found in the lumenal debris of all mice which had not been given antibiotics (See Fig. 6). In antibiotic-fed mice (See Fig. 7), greatly reduced

numbers of bacteria were seen in the lumen of the colon; since they were not recovered by culture, they were considered non-viable forms which had not yet been eliminated by defecation. Gram positive and Gram negative fungi were also found in the same location. No animal demonstrated fungal or bacterial invasion of the colon wall.

Irradiated mice, whether fed plain water or antibiotics, began to die on the eighth post-irradiation day (See Fig. 8). The mean survival time for irradiated mice was 10.7 days and for antibiotic-fed irradiated mice, 11.7 days. By the Kolmogorov-Smernov two-sample test, antibiotics did not produce a significant (p less than or equal to .05) prolongation of life (145). All irradiated animals died (See Fig. 9); among the unirradiated animals, there were no deaths for 30 days, and the study was terminated at that time.

DISCUSSION

The mice employed in these experiments were more resistant to x-radiation than those animals described in earlier studies. Previously, 1200 rads was followed by death in 4-5 days from the acute intestinal radiation syndrome (16-19); however, our mice survived, on the average, until eleven days after irradiation and demonstrated few, if any, gastrointestinal symptoms. Although clots, punctate hemorrhages, and dark stool were found in the gut at autopsy, there was no vomiting, diarrhea, melena, or dehydration. After a review of earlier histological studies (22-31), small bowel damage in the first three days after 900-1200 rads was much less than expected. Nuclear vacuolation, pyknosis, and the suppression of mitosis occurred as previously described, but there was less extensive disruption of cells and disintegration of tissues. The large bowel underwent a similar sequence of histologic changes; however, the slower and less severe nature of the damage was probably related to the colon's slower cell turnover rate (146). When death occurred, it was associated with the proliferation of colonic fecal bacteria at a time and in a manner consistent with the postirradiation bone marrow syndrome. Although the radioresistance of these mice is unexplained, it must be taken into consideration when comparing our results to those of previous investigators.

The mechanism by which the gastrointestinal tract recovers from radiation damage is not well understood. Biochemical studies have shown that intestinal DNA synthesis is suppressed during the first day after irradiation (69). Injected DNA is taken up by many tissues of the body, including the intestine; it is reported to prolong life and to reduce mortality in irradiated animals (122,123). Accordingly we undertook a study to discover whether the lifesaving effect of DNA was due to the amelioration of gastrointestinal radiation damage.

DNA was taken up by the jejunum, as shown by the mitotic overshoot in unirradiated mice at 2 hours after injection (See Fig. 3). However, DNA given just prior to irradiation or just after irradiation had no modifying effect on intestinal mitotic rate or intestinal damage in the first 48 hours after irradiation. No significant differences of tissue architecture, cell integrity, DNA localization and content, enzyme activity, or mitotic activity could be demonstrated in animals receiving DNA or saline and between animals receiving DNA before or after irradiation.

The lack of a gastrointestinal response to injected DNA does not preclude a beneficial effect on other organ systems. Indeed, spleen and bone marrow homogenates, for which DNA is the active principle, have proved effective against death from the bone marrow syndrome but

not against death from the acute intestinal radiation syndrome (147,148). Uptake studies show that 50% of the injected DNA is soon bound to blood cells, especially lymphocytes (122). Therefore, the lifesaving effects of DNA injections are more readily attributed to the enhanced recovery of lymphoid tissue and circulating leucocytes than to enhanced gastrointestinal recovery.

When Experiment I was performed, histochemical studies were considered superior to biochemical analyses for the localization of intestinal enzymes and nucleic acids. Biochemical studies were performed on whole organs or organ homogenates and, accordingly, measured the average activity of a tissue; these studies were unable to relate intestinal enzyme activity or nucleic acid content to a particular type of cell or to an individual cell. Histochemical techniques, on the other hand, have facilitated the qualitative localization of these compounds within the many component cells of the intestinal wall. However, it has been our experience and that of at least one previous investigator (149) that quantitative interpretation of histochemical preparations is subject to much error.

The quantitative assessment of a particular cell's enzyme activity requires the grading of its staining

intensity on microscopic examination. The comparison of tissue sections from many animals requires fine distinctions between the staining reactions of each, and it is questionable whether even an unbiased human eye is capable of such distinctions. Although some authors (27) have attempted to circumvent this problem by supposedly standardizing the treatment of all tissue sections and using photometric equipment, many variables (eg. fluctuations in the thickness of tissue sections, the efficacy of fixation for each preparation, and the variable rate of histochemical reactions) are unavoidable. Such attempts are conscientious, but the data is nonetheless unreliable.

With the inadequacy of histochemical techniques, it is not surprising that the Bacq-Alexander enzyme release theory has not been confirmed to date and that there is much conflicting opinion regarding the effect of radiation on intestinal enzymes. Our study demonstrated no persistent changes in enzyme activity in the first 48 hours after irradiation, but final comment should be deferred until better histochemical procedures are available.

Dubos, Schaedler, and others have demonstrated that the flora of the mouse digestive tract changes with the age of the animal (137,150). The rodent fetus is essentially free of cultivatable microorganisms at the moment of

birth; however, many bacterial species become established throughout the gastrointestinal tract with the onset of nursing. Lactobacilli, anerobic streptococci, and flavobacteria appear within the first day after birth and colonize the entire digestive tract. Lactobacilli and anerobic streptococci, according to these authors, are always more numerous in the stomach and colon than in the small intestine; they increase in number until a maximum level is reached on the twelfth day of the animal's life. From then on, as long as the animal is maintained under favorable physiologic conditions, these bacteria remain at nearly the same level. The flavobacteria also colonize the entire digestive tract and reach their maximum number around the tenth day of life; in contrast to lactobacilli and anerobic streptococci, they are most numerous in the small bowel. Their presence is transient, and they disappear completely by the twelfth day of life.

At this time, enterococci and slow lactose-fermenting coliforms proliferate rapidly in the colon (up to 10^9 organisms/gram of stool) and occasionally in the stomach and small bowel. However, this proliferation also is shortlived, and by the eighteenth day of life, their numbers have decreased to 10^3-10^4 organisms/gram, a level at which they persist as long as the animal remains under favorable physiological conditions. Bacteroides begin to proliferate exclusively in the

large intestine on the fifteenth day of life and multiply rapidly up to 10^9 organisms/gram, a level at which they persist throughout the animal's life. Thus, by the end of the third week of a healthy mouse's life, the colonic stool contains 10^{10} enterococci, and 10^4 coliforms per gram of specimen (137,150). This colonic flora remains remarkably constant, and that of the healthy adult mouse differs on some occasions only in the increased numbers of coliforms (10^8/gram), E. coli (10^7/gram), and occasional Pseudomonas, Proteus, or Clostridia (138).

Dubos and Schaedler have confirmed their bacteriologic data by histological studies of the stomach, small intestine, and colon (150). Through the use of the Brown-Brenn tissue Gram stain technique (143), they were able to demonstrate thick Gram positive rods where they recovered lactobacilli, Gram positive cocci where they recovered streptococci, small Gram negative rods where they recovered coliforms, and Gram negative fusiforms where they recovered bacteroides (150). In so doing, they destroyed the notion that gastrointestinal bacteria are randomly mixed throughout the gut and established the concept that the gut is a "collection of distinct microenvironments" in which virtually pure cultures of a few bacterial species exist (150). These bacteria interact with their host and adjacent microbial populations

in a series of changing temporal relationships. Thus, the irradiation of a weanling mouse, whose gastrointestinal flora is not firmly established, differs from the irradiation of an adult mouse with a stable bacterial composition.

Our mice differed from those of Dubos and Schaedler in the respect that fewer bacterial species were recovered. In no animal could we find anerobic streptococci; however, this may be entirely appropriate when the age of the mice is considered (137). Lactobacilli, total aerobes, total anerobes, bacteroides, and coliforms occurred in the same locations and proportions as those described by Dubos and Schaedler; but our recovery of bacteroides and total anerobes was slightly less than theirs. In only isolated instances did we recover Clostridia, flavobacteria, or Proteus. Thus, our baseline bacterial counts generally agreed with those of earlier studies (137,138,140), and any differences could be explained by the normal variation found among different strains of mice or among different colonies of the same strain.

In previous experiments of this type, statistical treatment of bacteriological data has been inaccurate or overzealous; normal variations (with existing techniques) of bacterial counts have been presented as statistically

significant events. However, the statistical significance of such data does not necessarily imply its bacteriological significance nor even its relevance to the experiment at hand. Before proceeding further with the discussion of results, it is necessary to review the difficulties with bacterial statistics.

The greater the number of organisms counted, the greater also will be the chance that insignificant variations in bacterial counts will be interpreted as significant. For example, if petri dish A had one bacterial colony on it, if petri dish B had two, and if both represented identically diluted specimens, no bacteriologist would seriously contend that these plates were different. If both plates were streaked from a 10^{-2} dilution, the variation represented by the one colony difference would be 100 organisms; if both plates were streaked from a 10^{-8} dilution, the actual bacterial counts would differ by 100 million organisms. By most statistical procedures, such a large number would be interpreted as a statistically significant increase of B over A. Thus, minor variations in bacterial recovery may be magnified when higher dilutions are involved, and a higher incidence of "false positives" (statistically significant changes) is produced. Indeed, this problem was encountered in the initial statistical treatment of our data, and a logarithmic transformation of actual

bacterial counts became necessary.

Through the use of logarithms, the number of bacterial colonies necessary to produce a statistically significant change remained the same for all dilutions of the original specimen. When the actual bacterial count doubled, the mantissa of the logarithm increased by .301 irrespective of the characteristic. The use of logarithms was also required by the tube dilution method of preparing the specimens for streaking. In any tube dilution procedure, a significant increase or decrease requires a two tube change in the reaction studied. In terms of this experiment, the actual bacterial count must increase or decrease by 100 fold for a bacteriologically significant event to have taken place. Changes of 1 tube (10 fold) or less are not bacteriologically significant, and conclusions derived from such data are not valid.

In the stampede to demonstrate bacterial overgrowth in the irradiated animal, many authors have erroneously accepted the validity of their statistical methods. One such study was published by Klainer, Gorbach, and Weinstein (136). These authors based many of their conclusions on poorly controlled experiments and insufficient data. All of their control bacterial counts were obtained in the week prior to irradiation, and statistical tests were based upon a comparison of these values with those of

irradiated animals 1-* weeks later. Because there were no unirradiated animals for comparison throughout the entire course of the experiment, these authors did not exclude the influence of extraneous factors (i.e., other than irradiation) on the bacterial counts of control and irradiated animals alike. Of the eighteen "significant" bacterial changes (Table 6 in reference 136) reported, thirteen were based upon variations of less than two powers of 10 (2 tube dilutions). Four of these 13 variations involved changes of 1.5-1.7 tube dilutions and were suggestive, but not truly significant. Sampling periods were infrequent, usually six to nine days apart, and two of the 13 variations were observed only on one occasion. Excluding unsubstantiated claims, these authors demonstrated proliferation of coliforms after 1050 rads, proliferation of coliforms and anerobic streptococci after 1550 rads, and proliferation of coliforms and fungi after 2150 rads.

Experiments II and III of this study were designed to avoid the unjustified acceptance of statistical significance as the equivalent of bacteriological significance. Untreated mice were used as controls throughout the entire experiment for comparison with irradiated animals. Sampling periods were frequent, usually every 2-3 days. Because of the many independent variables affecting bacterial counts, medium, presence or absence of radiation, presence or absence of antibiotics, and

time of sacrifice), a factorial analysis of variance was selected as the most suitable statistical test.

For each particular specimen, bacterial medium, and treatment group, mean bacterial counts were calculated each time of sacrifice. For each treatment group, a marginal mean bacterial count was obtained by averaging the means from each sampling period. The F ratio for the effect of radiation, defined as Mean Square Radiation/ Mean Square Error, was calculated to test the hypothesis that radiation had no effect on the marginal mean bacterial counts. The greater the F ratio, the less likely was the probability (p value) that this hypothesis was correct (151,152-Table B3).

Many difficulties were encountered even with this more sophisticated, computerized statistical analysis. In Experiments II and III combined, seventeen differences between the control and irradiated marginal mean bacterial counts were significant at the .01 level (See Tables 5 and 8); of these, twelve were due to extraneous factors. The F ratio compared the differences between control and irradiated groups of mice to the variation within each group. In some instances, the recovery of organisms in one group of mice (but not the other) was either nonexistent or so erratic that the comparison between animal groups was meaningless. In other instances, one group started the experiment with a higher count for

a particular organism and maintained this difference until the end without progression or variation. Often, the variation within a group was so slight that any differences between the groups produced significant F ratios. Thus, the factorial analysis of variance was superior in that it was based on bacterial counts throughout the entire duration of the experiment rather than on recovery at a single point in time; however, even this relatively sophisticated technique did not guarantee that a statistically significant event truly represented the bacteriologically significant result of a particular treatment.

As with previous statistical methods, the factorial analysis of variance included too many "false positives". In order to compensate for this disadvantage, it was necessary for us to apply stringent criteria for accepting a statistically significant event as real. To accept an increased bacterial count as a true radiation-induced proliferation, we required that: 1) irradiated animals have a marginal mean bacterial count 100 fold greater than that of controls; 2) the p value be .01 or less; and 3) the increased marginal mean bacterial count for irradiated animals be sustained for 2-3 sampling periods. Using such criteria, we demonstrated true increases in colonic fecal aerobes and coliforms of irradiated weanling mice and in colonic fecal aerobes, anerobes and coliforms of

irradiated adult mice; such increases occurred as agonal or pre-agonal events.

The proliferation of fecal coliforms and aerobes is consistent with the previously demonstrated involvement of Gram negative bacilli in postirradiation bacteremia (71-78,131). Fecal coliforms also multiply after high meat, gluten, or casein diets (105,153,154), starvation (153), some antibiotics (155), changes in environmental temperature, change of cages, fighting, laboratory manipulation, and other stresses (156). In such instances, and in the period after irradiation, the proliferation of coliforms has been associated with a suggested decrease in anerobic lactobacilli (136,156).

An antagonistic relationship between anerobic lactobacilli and coliforms has been observed by several authors (105,137,138,150,153-158) and has been attributed to an antibiotic-like substances, lactobacillin, secreted by some species of lactobacillus (159-162). The lactobacillus was thought to be the intestinal biostat which limited the size of other bacterial populations. According to this concept, disruptions of the gastrointestinal tract which produce conditions unfavorable for the growth of lactobacilli will be followed by an explosive proliferation of Gram negative species. Klainer, Gorbach, and Weinstein suggested that the fatal proliferation of coliforms which they demonstrated in irradiated animals

was due to a reduction in lactobacilli (136). Through dietary manipulation, these authors increased coliform growth and suppressed lactobacilli prior to irradiation; the animals died more rapidly and in greater numbers (154). Surprisingly, when lactobacilli were increased by dietary manipulation, there was no protection against irradiation (154); in one study, increased numbers of lactobacilli were associated with increased postirradiation mortality (163).

The significance of the agonal increase in Gram negative bacilli remains unclear. There is no question that such overgrowth is related to bacteremia and greater mortality; however, the pathogenesis of this relationship is still open to debate. By two independent techniques we demonstrated that massive bacterial invasion of the intestinal wall does not take place. Bacterial counts in tissue homogenates (See Tables 3c, 3f, and 3j) remained stable throughout the course of the experiment, and bacteria were seen only at the lumenal surface of the gut.

Instead of a massive invasion, Osborne (17), Bond (18), Gordon (81), and others have suggested that small numbers of bacteria cross the epithelial barrier at the time of maximal villous damage. The spread of the organisms is limited at first to small clumps of bacteria near submucosal lymphatics and capillaries. When immunological defenses fail in the second week after

irradiation, bacteria may be found in regional lymph nodes, liver, and spleen (79). These organs which serve as a secondary line of defense, are now incompetent and no longer prevent hematogenous dissemination of bacteria. Thus, only a small number of organisms are sufficient to produce fatal bacteremia.

Osborne also postulated that bacteremia and death could be related to coprophagy. Mice and other animals are frequently infected by the fecal-oral route. When the host animals defense mechanisms are destroyed, ingested bacteria may also enter the bloodstream by way of the tonsils and cervical lymphatics (17).

Other workers implicated endotoxemia and vascular collapse as the mechanism of death. They postulated that increased numbers of Gram negative bacteria in the stool resulted in a greater production of endotoxin. Although data is conflicting, irradiated mice are more susceptible to any quantity of endotoxin (88,131). The susceptibility of these animals to the lethal effects of endotoxin usually requires previous sensitization to Gram negative bacteria; this could occur when coliform bacilli multiply explosively in the 10 day old mouse prior to equilibration with the lactobacillus population (137,156). The rapidly progressing pallor, prostration, and death is further evidence for endotoxic shock as an important mechanism of death in the second week after irradiation.

Matsuzawa, Wilson, and others who have worked with

germfree mice postulate a less direct effect of intestinal bacteria on postirradiation survival. Germfree mice live longer than conventional mice after "bone marrow syndrome" doses of radiation. This finding was attributed to the fact that, in germfree animals, incompetent immunologic defense mechanisms are not exposed to intestinal bacteria (20,132,133). However, after "intestinal syndrome" doses of radiation, when death supposedly is unrelated to infectious processes, germfree mice continue to outlive conventional mice (19,164). In explanation, these authors have shown that the presence of intestinal bacteria increases the mitotic rate and shortens the lifespan of intestinal epithelial cells (165,167). Because cellular radiosensitivity is proportional to mitotic rate, intestinal bacteria may influence gastrointestinal damage by their effort on cell turnover rate.

Invariably, all proposed mechanisms of radiation death in the second week after exposure mention the impairment of host defenses. Because the host animal is immunologically incompetent, it is unable to contain or regulate any of the microorganisms which it harbors. It is susceptible to small numbers of bacteria or to relatively avirulent organisms (82,86). Even the complete elimination of recoverable bacteria from the gut and the substitution of noninvasive fungi does not necessarily prolong life or reduce mortality. Thus,

although pathological alterations occur in the gastrointestinal flora, it is the amount of damage to the host animal's protoplasm which ultimately determines survival.

For this reason, postirradiation sickness is not a single entity; it is the composite of a number of syndromes which reflect a wide spectrum of cellular radiosensitivity (168). Each cell population of the body may be characterized by its degree of radiosensitivity. As the exposure to radiation increases, the threshhold for irreversible damage is exceeded for a greater number of tissues. Lymphoid and hemopoietic organs contain the most radiosensitive cells of the body. At dosages below the LD_{50}, damage to these organs is reversible; antibiotics prevent death from infection until immunologic recovery takes place (130). At LD_{50} dosages, lymphoid damage is irreversible, antibiotics will not prevent death, and some form of replacement therapy was impractical, and death was inevitable after this amount of damage. However, the prolongation of life and the reduction of mortality following injections of bone marrow homogenates, spleen homogenates, or DNA in large doses (110-114,122) has prompted investigators to reconsider these treatments in the control of radiation sickness and death.

As radiation exposure nears LD_{100} dosages, rever-

sible damage to the gastrointestinal epithelium takes place. Therapy here requires all of the previous treatments plus the control of diarrhea and the careful attention to fluids, electolytes, and acid-base balance. At higher doses of radiation, gastrointestinal damage is irreversible, and replacement therapy for this organ system is necessary. At present, no known drug therapy will restore the intestinal epithelium. For the future one potential mode of therapy for this amount of damage is total intestinal transplant.

At subsequently higher doses of radiation, damage involves so many organ systems and death occurs so rapidly that transplantation is out of the question. At this point, and perhaps at lower dosages, the ideal therapy for radiation exposure would attack a basic and universal cytological lesion. Ultimately, "cure" for radiation sickness and radiation death will depend on the stimulation of intracellular recovery processes and the prophylaxis of subcellular radiation damage.

SUMMARY

The morphological, histochemical, and bacteriological effects of ionizing radiations on the gastrointestinal tract of mice are reviewed in this paper; their relationship to postirradiation sickness and death is discussed. Histochemical techniques, bacteriological techniques, experimental designs, and statistical methods used in this paper and previous studies are presented and analyzed.

Intraperitoneal DNA, which prolongs life and reduces mortality in irradiated animals, had no effect on gastronintestinal damage. Others have attributed its beneficial effects to the enhanced regeneration of damaged hemopoietic and immunologic mechanisms.

Bacteria proliferate in the colonic feces of irradiated mice, but they do not invade the surrounding tissues of the intestinal wall. The suppression of these bacteria by antibiotics did not prolong life or reduce mortality after a "hemopoietic failure" dose of x-radiation. Accordingly, it was concluded that for the ultimate survival of the host animal, the extent of tissue damage is more important than bacterial proliferation.

POSTIRRADIATION INTESTINAL ENZYME ACTIVITY

Key to Animal Treatment Groups

O	- no treatment
S	- saline injection, sacrificed without irradiation
D	- DNA injection, sacrificed without irradiation
I&D	- irradiation followed by DNA injection
I&S	- irradiation followed by saline injection
D&I	- irradiation preceded by DNA injection

Enzyme activity, which is represented by the intensity of the histochemical stain, has been graded from 0 (absent) to 4.

Hepatic Cathepsin Activity - control specimen

Table 1a

Unirradiated Mice

Treatments		
O	S	D
3	3	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	3	4	3	3
2 hrs.	3	3	2	2
4 hrs.	3	2	2	2
8 hrs.	2	3	3	2
24 hrs.	2	2	2	2
48 hrs.	3	3	3	2

Jejunal Cathepsin Activity at the Villus tip

Table 1b

Unirradiated Mice

Treatments		
O	S	D
2	3	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	3	3	4	4
2 hr.	2	4	4	3
4 hr.	3	3	1	2
8 hr.	3	3	2	2
24 hr.	3	2	3	2
48 hr.	2	3	4	3

Jejunal Cathepsin Activity at the Villous side

Table 1c

Unirradiated Mice

Treatments		
O	S	D
2	2	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	2	3	4	3
2 hrs.	2	3	4	3
4 hrs.	3	2	3	2
8 hrs.	3	3	2	2
24 hrs.	3	2	3	2
48 hrs.	2	3	4	2

Jejunal Cathepsin Activity at the Villous base

Table 1d

Unirradiated Mice

Treatments		
O	S	D
0	0	0

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	1	3	1
2 hr.	1	2	2	2
4 hr.	2	1	2	1
8 hr.	1	2	1	1
24 hr.	1	1	1	1
48 hr.	1	2	1	1

Jejunal Cathepsin Activity at the Crypt glands

Table 1e

Unirradiated Mice

Treatments		
O	S	D
0	0	0

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	0	0	1	1
2 hrs.	0	1	0	1
4 hrs.	1	1	0	0
8 hrs.	0	0	0	0
24 hrs.	0	0	0	0
48 hrs.	0	0	0	0

Ileal Cathepsin Activity at the Villus tip

Table 1f

Unirradiated Mice

Treatments		
O	S	D
1	1	1

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	2	1	2
2 hr.	1	2	2	3
4 hr.	2	2	2	2
8 hr.	2	1	2	2
24 hr.	3	1	1	3
48 hr.	1	2	3	2

Ileal Cathepsin Activity at the Villus side

Table 1g

Unirradiated Mice

Treatments		
0	S	D
1	1	1

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	1	1	2
2 hrs.	1	2	2	3
4 hrs.	2	2	2	2
8 hrs.	2	1	2	1
24 hrs.	3	1	1	2
48 hrs.	1	2	2	3

Ileal Cathepsin Activity at the Villus base

Table 1h

Unirradiated Mice

Treatments		
0	S	D
0	0	0

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	0	1	1	1
2 hr.	0	1	1	1
4 hr.	1	1	1	1
8 hr.	2	0	1	1
24 hr.	1	0	0	0
48 hr.	0	1	0	0

Ileal Cathepsin Activity at the Crypt glands

Table 1j

Unirradiated Mice

Treatments		
O	S	D
0	0	0

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	0	0	0	0
2 hrs.	0	0	0	0
4 hrs.	0	0	0	0
8 hrs.	0	0	0	0
24 hrs.	0	0	0	0
48 hrs.	0	0	0	0

Hepatic Nonspecific Esterase Activity - control specimen

Table 2a

Unirradiated Mice

Treatments		
O	S	D
2	3	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	2	3	2	2
2 hr.	2	2	2	2
4 hr.	2	2	0	1
8 hr.	1	1	2	2
24 hr.	2	2	3	1
48 hr.	2	2	2	2

Jejunal Nonspecific Esterase Activity at the Villus tip

Table 2b

Unirradiated Mice

Treatments		
O	S	D
3	3	3

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	3	2	3	2
2 hrs.	3	2	4	3
4 hrs.	3	3	3	2
8 hrs.	2	2	3	3
24 hrs.	2	3	4	1
48 hrs.	2	2	1	2

Jejunal Nonspecific Esterase Activity at the Villus side

Table 2c

Unirradiated Mice

Treatments		
O	S	D
3	3	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	2	2	3	2
2 hr.	2	2	4	2
4 hr.	2	3	3	2
8 hr.	2	2	3	2
24 hr.	2	3	4	1
48 hr.	1	2	4	2

Jejunal Nonspecific Esterase Activity at the Villus base

Table 2d

Unirradiated Mice

Treatments		
O	S	D
2	2	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	2	3	3	2
2 hrs.	2	2	4	1
4 hrs.	2	2	2	2
8 hrs.	1	2	3	2
24 hrs.	1	3	4	1
48 hrs.	1	1	3	1

Jejunal Nonspecific Esterase Activity at the Crypt glands

Table 2e

Unirradiated Mice

Treatments		
O	S	D
1	1	1

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	2	2	1
2 hr.	1	1	1	1
4 hr.	1	1	1	1
8 hr.	1	1	2	1
24 hr.	1	2	2	1
48 hr.	1	1	1	1

Ileal Nonspecific Esterase Activity at the Villus tip

Table 2f

Unirradiated Mice

Treatments		
O	S	D
2	2	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	2	2	3
2 hrs.	2	2	2	3
4 hrs.	2	3	2	3
8 hrs.	2	2	3	2
24 hrs.	3	3	3	2
48 hrs.	2	3	4	4

Ileal Nonspecific Esterase Activity at the Villus side

Table 2g

Unirradiated Mice

Treatments		
O	S	D
2	2	2

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	3	2	2
2 hr.	2	2	3	3
4 hr.	2	2	2	3
8 hr.	2	2	3	2
24 hr.	2	3	3	1
48 hr.	2	3	4	4

Ileal Nonspecific Esterase Activity at the Villus base

Table 2h

Unirradiated Mice

Treatments		
O	S	D
2	1	1

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	2	2	1
2 hrs.	1	1	3	3
4 hrs.	1	1	1	2
8 hrs.	1	1	2	1
24 hrs.	1	2	2	1
48 hrs.	1	3	4	3

Ileal Nonspecific Esterase Activity at the Crypt Glands

Table 2j

Unirradiated Mice

Treatments		
O	S	D
0	0	0

Irradiated Mice

Time	I&D	I&S	D&I	D
1 hr.	1	0	1	1
2 hr.	0	1	0	0
4 hr.	1	0	0	1
8 hr.	1	1	0	0
24 hr.	0	0	0	0
48. hr.	1	1	1	1

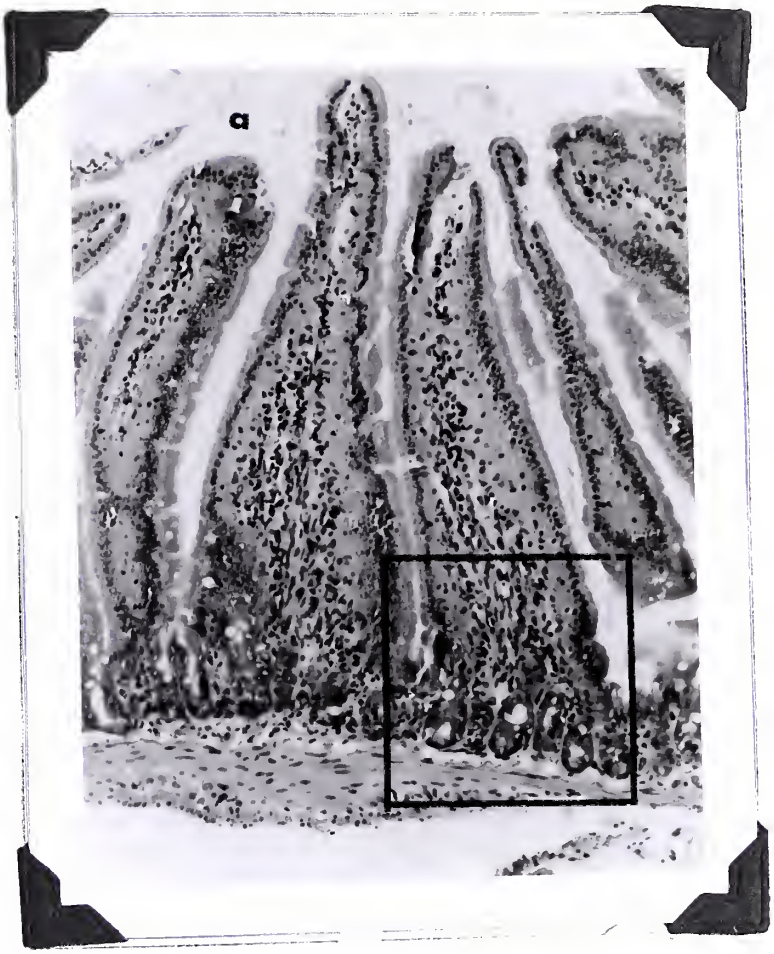
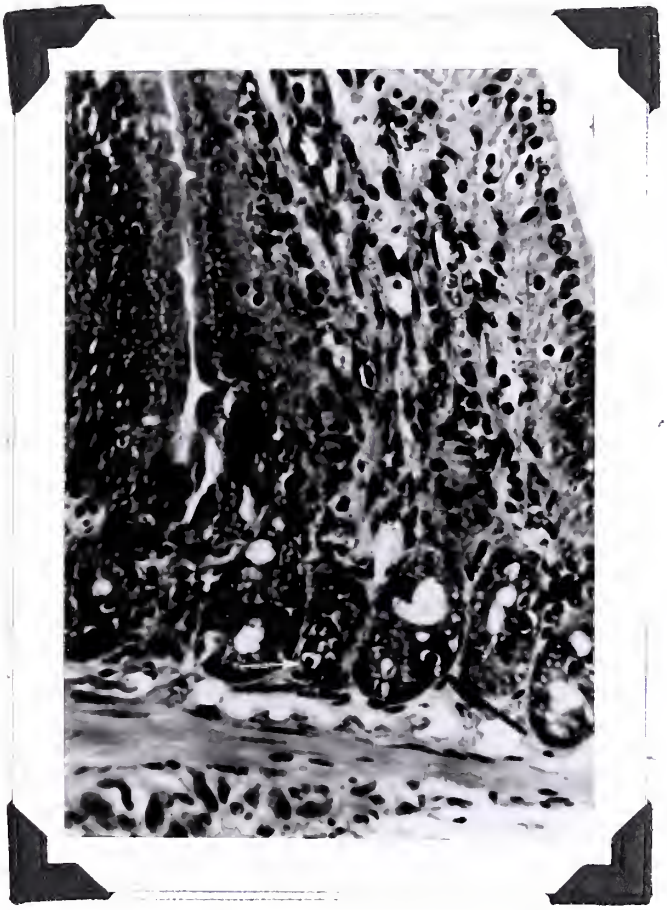


FIG. 1.



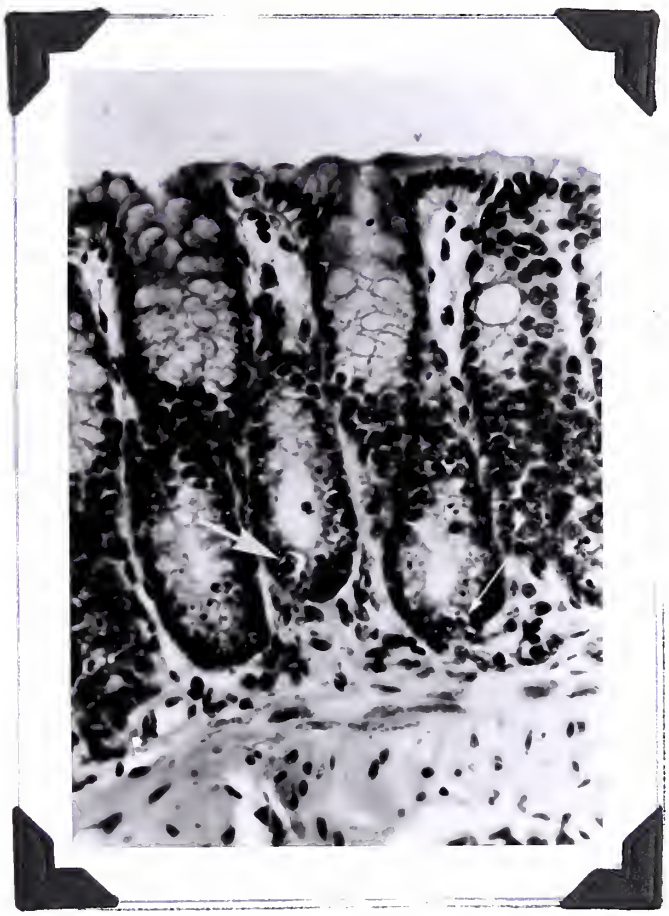
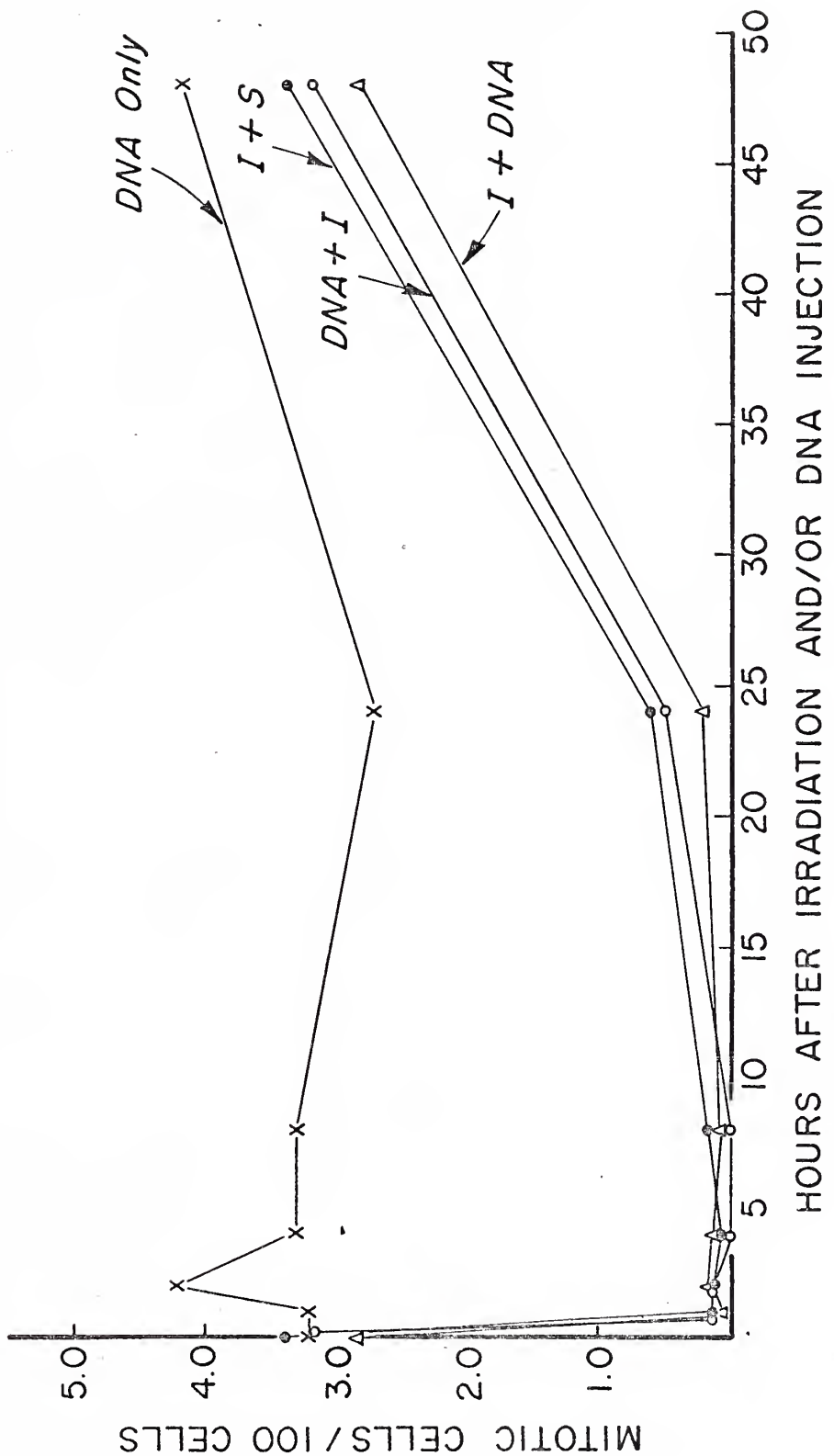


FIG. 2.

Fig. 3


DNA Only
I + S
DNA + I
I + DNA
MITOTIC CELLS / 100 CELLS
5.0
4.0
3.0
2.0
1.0
HOURS AFTER IRRADIATION AND/OR DNA INJECTION
5
10
15
20
25
30
35
40
45
50

Legends

FIG. 1

a). Low power photomicrograph of small bowel from mouse sacrificed four hours after irradiation. The villus height appears normal, but note b) which is a high power photomicrograph of outlined insert in a). There is an increase in round cell infiltrate and the arrows point to damaged cells in the crypts that have become vacuolated and contain inclusion bodies. (Reproduction Ratio X100 and X400).

FIG. 2

High power photomicrograph of colon of mouse sacrificed eight hours after irradiation. Note the disorientation of nuclear material in the crypt cells. The arrows point to inclusion bodies within vacuoles in damaged cells. (Reproduction Ratio X400).

FIG. 3

Graphic representation of the relationship between small bowel crypt cell mitotic and hours after irradiation (I). Note the depression of mitotic counts after irradiation regardless of whether the animals received DNA before (DNA + I) or after (I + DNA) irradiation or irradiation plus saline (I + S).

Mean Bacterial Counts (Log bacteria/gram specimen) in the Stomach Contents of Weanling Mice

Table 3a

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	4.30	6.45	7.35	4.00	3.00	1.00	7.30	4.77
	Con.	6.23	7.60	7.00	7.00	5.30	1.00	5.15	5.61
A-	Irr.	7.54	9.13	8.09	8.68	7.89	6.62	8.15	8.01
	Con.	8.84	8.79	8.65	8.65	8.97	6.70	8.58	8.45
C	Irr.	1.00	4.24	1.00	2.74	1.00	1.00	6.81	2.54
	Con.	6.04	6.00	1.00	1.00	4.60	1.00	4.78	3.49
E	Irr.	1.00	2.65	1.00	1.00	1.00	1.00	1.00	1.57
	Con.	1.00	5.00	1.00	1.00	1.00	1.00	1.00	1.57
G	Irr.	7.62	9.00	8.98	8.64	7.80	5.87	10.53	8.35
	Con.	8.00	9.00	8.81	8.81	8.08	8.34	10.04	8.73
Ent	Irr.	7.04	9.04	8.79	9.15	7.84	6.19	7.90	7.99
	Con.	8.49	8.45	8.64	8.64	7.04	7.48	8.85	8.23

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/cc. specimen in the Stomach Wash of Weanling Mice

Table 3b

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	3.35	6.29	5.80	3.80	2.35	1.00	4.00	3.80
	Con.	6.65	6.54	5.70	5.70	1.00	1.00	4.00	4.37
A-	Irr.	6.65	8.95	6.52	7.70	6.42	5.83	7.70	7.18
	Con..	8.18	8.18	6.98	6.98	7.49	5.28	8.00	7.30
C	Irr.	1.00	3.06	1.00	2.50	1.00	1.00	6.18	2.25
	Con.	4.30	4.60	1.00	1.00	1.00	1.00	4.00	2.41
E	Irr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Con.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
G	Irr.	6.88	9.07	7.85	8.57	7.22	6.42	7.20	7.60
	Con.	6.00	8.48	8.65	8.65	8.00	5.70	8.00	7.64
Ent	Irr.	5.44	8.18	7.46	7.87	8.08	3.77	7.20	6.86
	Con.	6.85	7.85	8.09	8.18	7.94	6.60	7.51	7.57

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/gram specimen) in the Stomach Homogenate of Weanling Mice

Table 3c

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	1.00	4.42	5.95	1.00	2.42	1.00	6.63	3.20
	Con.	7.00	5.73	3.83	3.83	3.89	1.00	4.43	4.24
A-	Irr.	5.11	8.22	7.71	6.67	7.29	6.35	8.30	7.09
	Con.	8.59	7.08	7.11	7.11	8.15	7.00	6.83	7.41
C	Irr.	2.50	1.00	1.00	1.00	1.00	1.00	4.89	1.77
	Con.	8.43	5.34	1.00	1.00	3.18	1.00	5.20	3.59
E	Irr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Con.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
G	Irr.	5.43	8.47	7.83	6.34	7.21	8.06	7.85	7.31
	Con.	8.00	7.52	8.43	8.43	8.18	8.08	9.43	8.30
Ent	Irr.	5.20	7.98	8.15	4.33	7.27	5.33	6.85	6.44
	Con.	8.00	7.78	7.30	7.30	8.18	6.08	9.11	7.68

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/gram specimen) in the Jejunal Contents of Weanling Mice

Table 3d

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	6.00	6.50	6.21	4.30	1.00	6.93	7.30	5.46
	Con.	6.30	1.00	5.30	5.30	1.00	4.00	5.78	4.10
A-	Irr.	7.00	8.78	7.87	7.45	7.74	7.09	8.00	7.70
	Con.	7.38	7.60	6.15	6.15	8.23	4.00	7.90	6.77
C	Irr.	1.00	1.00	1.00	1.00	2.89	1.00	1.00	1.27
	Con.	1.00	1.00	1.00	2.80	1.00	1.00	1.00	1.26
E	Irr.	4.60	1.00	1.00	1.00	1.00	3.71	1.00	1.90
	Con.	4.60	1.00	1.00	1.00	1.00	1.00	1.00	1.51
G	Irr.	1.00	8.95	7.84	7.45	7.15	7.31	8.30	6.86
	Con.	1.00	7.60	8.30	8.30	8.72	6.48	8.60	7.00
Ent	Irr.	7.78	9.00	8.17	7.94	7.49	3.42	8.30	7.44
	Con.	7.78	7.30	8.58	8.53	8.56	4.85	6.64	7.47

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/cc specimen) in the Jejunal Wash of Weanling Mice

Table 3e

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	1.00	3.50	2.65	2.85	3.35	4.90	2.90	3.02
	Con.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A-	Irr.	3.18	6.85	5.57	5.70	5.39	4.93	5.70	5.33
	Con.	6.60	8.00	4.95	4.95	5.74	3.70	6.00	5.71
C	Irr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Con.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
E	Irr.	1.00	1.00	2.80	1.00	1.00	1.00	1.00	1.26
	Con.	1.00	5.70	1.00	1.00	1.00	1.00	1.00	1.67
G	Irr.	3.57	7.80	6.05	5.63	6.26	5.49	7.70	6.07
	Con.	4.79	6.99	6.17	5.96	6.33	5.28	6.81	6.07
Ent	Irr.	3.10	6.76	5.78	5.57	6.45	2.59	6.23	5.07
	Con.	6.00	6.40	6.65	6.65	6.48	1.00	5.81	5.57

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/gram specimen) in the Jejunal Homogenate of Weanling Mice

Table 3f

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	4.63	7.81	1.00	2.89	2.41	6.18	4.96	4.27
	Con.	5.51	1.00	3.77	3.77	3.70	1.00	1.00	2.82
A-	Irr.	5.33	7.03	5.76	4.91	7.00	5.91	6.96	6.13
	Con.	6.38	6.75	5.77	5.77	5.65	5.78	6.20	6.04
C	Irr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Con.	4.60	1.00	1.00	1.00	5.40	1.00	1.00	2.14
E	Irr.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	Con.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
G	Irr.	5.24	7.37	6.03	4.99	5.02	5.85	6.15	5.81
	Con.	6.15	5.65	5.72	5.72	6.85	7.88	6.08	6.29
Ent	Irr.	5.45	6.93	6.14	4.53	5.63	4.09	6.28	5.58
	Con.	6.67	6.73	5.61	5.61	6.70	5.45	6.00	6.11

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/gram specimen) in the Colon Contents of Weanling Mice

Table 3g

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	6.45	7.66	7.89	8.50	7.03	9.07	8.00	7.80
	Con.	7.00	8.38	7.00	7.00	5.53	5.30	5.60	6.54
A-	Irr.	7.22	8.63	8.32	8.66	8.02	8.69	9.38	8.42
	Con.	9.15	8.85	8.00	8.00	8.34	8.28	8.48	8.44
C	Irr.	7.13	6.34	1.00	7.92	5.31	3.60	8.08	5.63
	Con.	6.92	6.53	1.00	1.00	6.86	4.30	7.90	4.93
E	Irr.	1.00	1.00	1.00	5.81	5.53	7.75	1.00	3.30
	Con.	1.00	1.00	1.00	1.00	7.00	4.00	4.90	2.84
G	Irr.	7.00	8.66	8.76	8.68	8.69	9.54	9.82	8.74
	Con.	8.74	8.69	8.78	8.78	8.30	9.00	8.78	8.72
Ent	Irr.	7.00	8.65	8.60	8.32	7.95	7.15	9.11	8.11
	Con.	9.28	8.70	8.30	8.30	7.48	7.00	8.41	8.21

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/ cc. specimen) in the Colon Wash of Weanling Mice

Table 3h

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	3.50	5.33	2.96	6.09	2.70	7.24	4.00	4.55
	Con.	1.00	1.00	5.51	5.51	1.00	1.00	1.00	2.29
A-	Irr.	7.32	5.85	5.02	5.81	5.24	6.85	5.70	5.97
	Con.	4.65	6.30	7.70	7.70	5.23	5.70	5.26	6.08
C	Irr.	4.41	1.00	1.00	1.00	1.00	1.00	1.00	1.49
	Con.	1.00	2.59	1.00	1.00	4.93	3.70	5.11	2.76
E	Irr.	1.00	1.00	1.00	2.59	1.00	5.63	1.00	1.84
	Con.	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
G	Irr.	7.03	6.48	5.85	3.95	6.09	5.55	5.18	5.73
	Con.	6.00	6.18	6.70	6.70	6.00	6.40	4.93	6.13
Ent	Irr.	7.21	7.24	5.65	5.69	6.15	1.00	5.26	5.46
	Con.	5.59	6.18	6.30	6.30	4.93	4.00	4.90	5.46

Irr. = Irradiated Mice
Con. = Control Mice

Mean Bacterial Counts (Log bacteria/gram specimen) in the Colon Homogenate of Weanling Mice

Table 3j

Media		Time of Sacrifice: 1 hr	1 day	2 days	3 days	5 days	7 days	10 days	Mean
A+	Irr.	4.41	6.07	3.12	1.00	5.87	7.89	4.40	4.68
	Con.	4.78	1.00	1.00	1.00	1.00	1.00	3.88	1.95
A-	Irr.	5.99	6.78	6.05	5.00	6.87	6.50	7.11	6.34
	Con.	6.41	4.73	6.72	6.72	5.85	5.99	6.88	6.19
C	Irr.	1.00	2.74	1.00	1.00	1.00	1.00	5.28	1.86
	Con.	1.00	4.60	1.00	1.00	1.00	1.00	5.36	2.14
E	Irr.	1.00	1.00	1.00	1.00	1.00	6.21	1.00	1.74
	Con.	4.30	1.00	1.00	1.00	1.00	1.00	1.00	1.47
G	Irr.	6.60	8.78	5.78	4.14	6.26	7.22	6.58	6.48
	Con.	6.00	4.83	6.26	6.26	6.78	6.23	6.18	6.07
Ent	Irr.	6.39	7.27	6.32	1.00	5.93	1.00	5.84	4.82
	Con.	6.00	4.83	6.23	6.23	5.48	1.00	5.63	5.06

Irr. = Irradiated Mice
Con. = Control Mice

F Ratios (Mean Square Treatment/Mean Square Error) for the Effect of Radiation on Bacterial Counts in Weanling Mice

Table 4

Specimen	Media A+	A-	C	E	G	Ent
Stomach Contents	2.17	24.97	3.26	2.03	7.80	2.65
Stomach Wash	0.95	0.31	0.21	0.00	0.02	1.53
Stomach Homogenate	18.30	1.29	23.26	0.00	8.06	43.39
Jejunum Contents	7.59	35.05	0.00	1.00	0.80	0.01
Jejunum Wash	11.13	1.31	0.00	2.59	0.01	1.54
Jejunum Homogenate	13.77	0.22	0.00	0.00	2.74	1.30
Colon Contents	25.17	0.03	1.87	8.95	0.01	0.33
Colon Wash	18.02	0.24	30.82	7.47	0.85	0.00
Colon Homogenate	50.98	0.85	1.25	0.78	1.38	1.70

Significance Levels (p values) for the Effects of Radiation on Bacterial Counts in Weanling Mice

Table 5

Specimen	Media A+	A-	C	E	G	Ent
Stomach Contents	$< .25$	$< .01$	$< .10$	$< .25$	$< .05$	$< .25$
Stomach Wash	$> .25$	$> .25$	$> .25$	$> .25$	$> .25$	$< .25$
Stomach Homogenate	$< .01$	$> .25$	$< .01$	$> .25$	$< .05$	$< .01$
Jejunum Contents	$< .05$	$< .01$	$> .25$	$> .25$	$> .25$	$> .25$
Jejunum Wash	$< .01$	$> .25$	$> .25$	$< .25$	$> .25$	$< .25$
Jejunum Homogenate	$< .01$	$> .25$	$< .01$	$> .25$	$< .25$	$> .25$
Colon Contents	$< .01$	$> .25$	$< .25$	$< .01$	$> .25$	$> .25$
Colon Wash	$< .01$	$> .25$	$< .01$	$< .05$	$> .25$	$> .25$
Colon Homogenate	$< .01$	$> .25$	$> .25$	$> .25$	$> .25$	$< .25$

Mean Bacterial Counts (Log bacteria/gram specimen) in Colonic Feces of Normally-Fed Adult Mice

Table 6a

Media		Time of Sacrifice: 1 day	3 days	5 days	7 days	10 days	Mean
A+	Con.	5.50	5.95	6.24	6.15	7.08	6.18
	Irr.	4.00	6.32	7.45	7.05	10.05	6.97
A-	Con.	8.02	6.50	8.42	7.57	8.30	7.76
	Irr.	7.44	8.75	8.50	9.02	10.14	8.78
C	Con.	7.06	7.07	7.48	6.80	2.00	6.08
	Irr.	7.44	8.78	8.26	7.42	8.96	8.27
E	Con.	5.00	4.87	5.92	4.90	5.45	5.23
	Irr.	3.15	5.98	7.48	6.50	10.23	6.67
G	Con.	8.31	7.62	8.08	8.15	8.08	8.05
	Irr.	8.17	8.69	8.47	8.76	9.48	8.72
Ent	Con.	8.16	6.80	8.24	8.54	8.30	8.01
	Irr.	8.45	9.06	8.62	7.97	9.17	8.65

Con. = Control Mice
Irr. = Irradiated Mice

Mean Colony Counts (Log fungi/gram specimen) in Colonic Feces of Antibiotic-Fed Adult Mice

Table 6b

Media		Time of Sacrifice: 1 day	3 days	5 days	7 days	10 days	Mean
A+	Con.	1.00	6.31	7.35	6.00	7.75	5.68
	Irr.	1.00	6.00	7.52	7.45	5.68	5.53
A-	Con.	1.00	5.91	7.33	6.25	7.54	5.61
	Irr.	1.00	5.87	9.00	7.84	5.75	5.89
C	Con.	1.00	6.16	7.75	6.12	7.76	5.76
	Irr.	1.00	6.19	6.00	7.25	6.10	5.31
E	Con.	1.00	6.07	6.81	5.95	7.36	5.44
	Irr.	1.00	6.00	5.96	6.81	3.42	4.64
G	Con.	1.00	4.59	4.00	2.00	6.00	3.52
	Irr.	1.00	6.90	7.93	6.00	5.59	5.48
Ent	Con.	1.00	2.00	2.00	2.00	2.00	1.80
	Irr.	1.00	2.00	3.85	2.00	2.00	2.17

Con. = Control Mice
Irr. = Irradiated Mice

F Ratios (Mean Square Treatment/Mean Square Error) for the Effect of Radiation on Bacterial Counts in Normally Fed Adult Mice and Fungal Counts in Antibiotic Fed Adult Mice

Table 7

	Media					
	A+	A-	C	E	G	Ent
Bacteria	12.05	12.26	45.58	14.34	5.56	2.16
Fungi	0.13	0.94	2.54	3.72	5.92	1.00

Significance Level (p values) for the Effect of Radiation on Bacterial Counts in Normally Fed Adult Mice and Fungal Counts in Antibiotic Fed Adult Mice

Table 8

	Media					
	A+	A-	C	E	G	Ent
Bacteria	$< .01$	$< .01$	$< .01$	$< .01$	$< .05$	$< .25$
Fungi	$> .25$	$> .25$	$< .25$	$< .10$	$< .05$	$> .25$

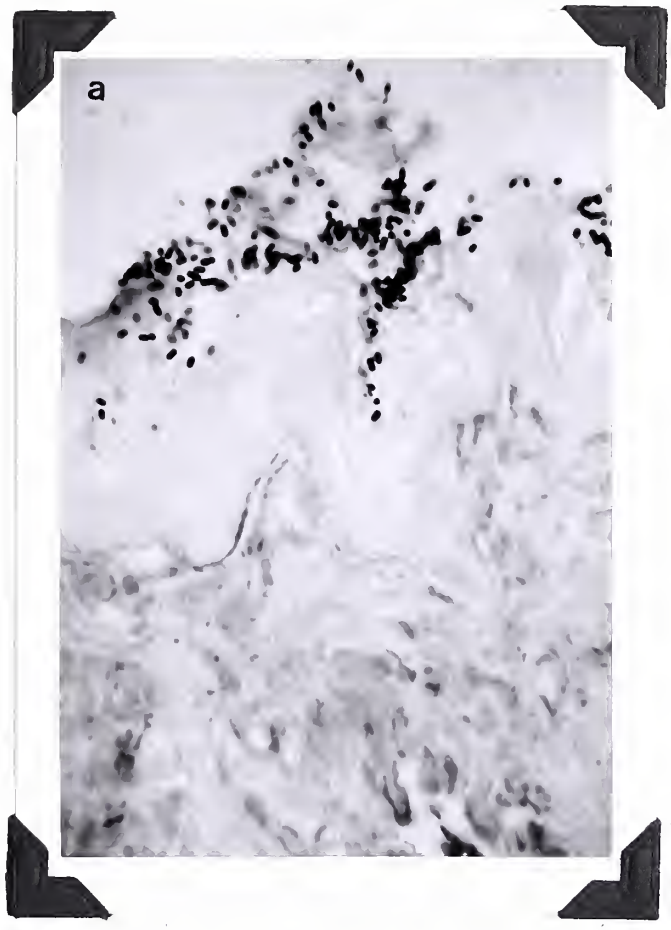
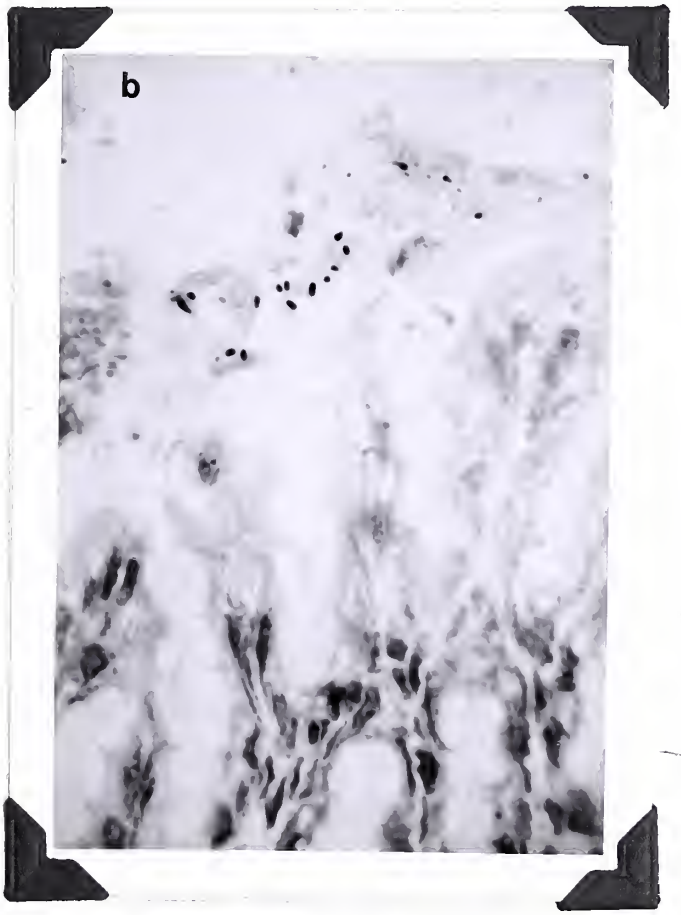


FIG. 4



Legends

FIG. 4

a). Oil immersion photomicrograph of a nonglandular area of stomach from an unirradiated weanling mouse. Brown-Brenn stain. Note the short pleomorphic gram-positive rods which are closely adherent to the stratified squamous epithelium and the mucus overlying it. (Reproduction ratio X960).

b). Oil immersion photomicrograph of a glandular area of stomach from a weanling mouse sacrificed one day after irradiation. Brown-Brenn stain. Glandular architecture is intact, although mucus production has increased. Gram-positive organisms are found within the mucus layer overlying the gastric glands. (Reproduction ratio X960).

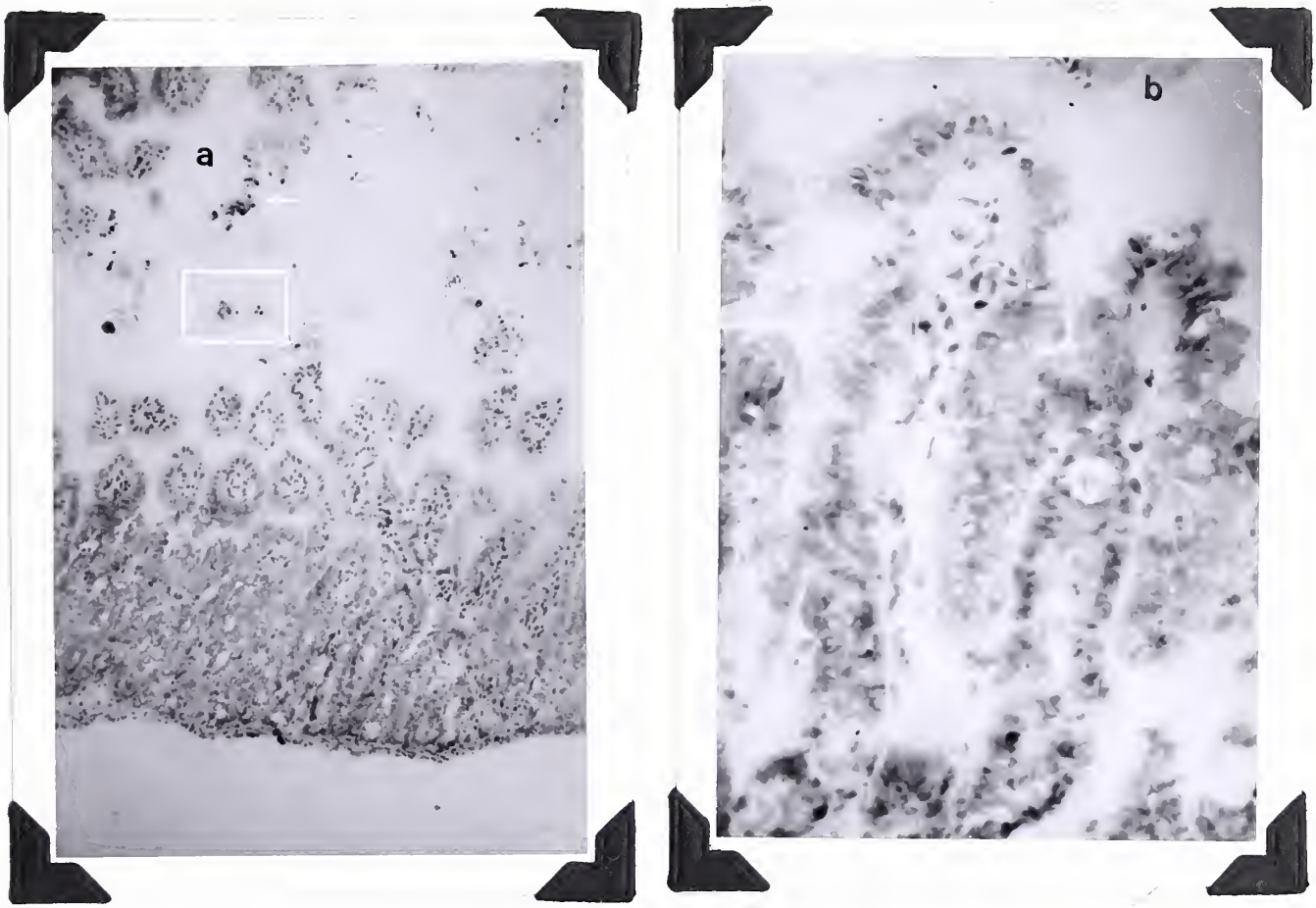


FIG. 5

Legends

FIG. 5

a). Low power photomicrograph of jejunum from a weanling mouse sacrificed three days after irradiation. Brown-Brenn stain. Villi are short, stubby, and edematous; damaged cells in the crypts are vacuolated and contain inclusion bodies. Compare with FIG. 1a. Clumps of bacteria (white arrows, white outlined insert) are seen in the lumen but not in any of the damaged tissues. (Reproduction ratio x100).

b). High power photomicrograph of a typical villus in FIG. 5a. Brown-Brenn stain. Bacteria are not closely related to the epithelial lining as they are in the stomach; compare with FIG. 4b. Although the villus is extensively damaged 3 days after irradiation, it has not been invaded by bacteria from the lumen. (Reproduction ratio x400).

c). Oil immersion photomicrograph of the clump of bacteria seen in the outlined insert of FIG. 5a. Short, pleomorphic gram-positive rods are the predominant organism. (Reproduction ratio x960).

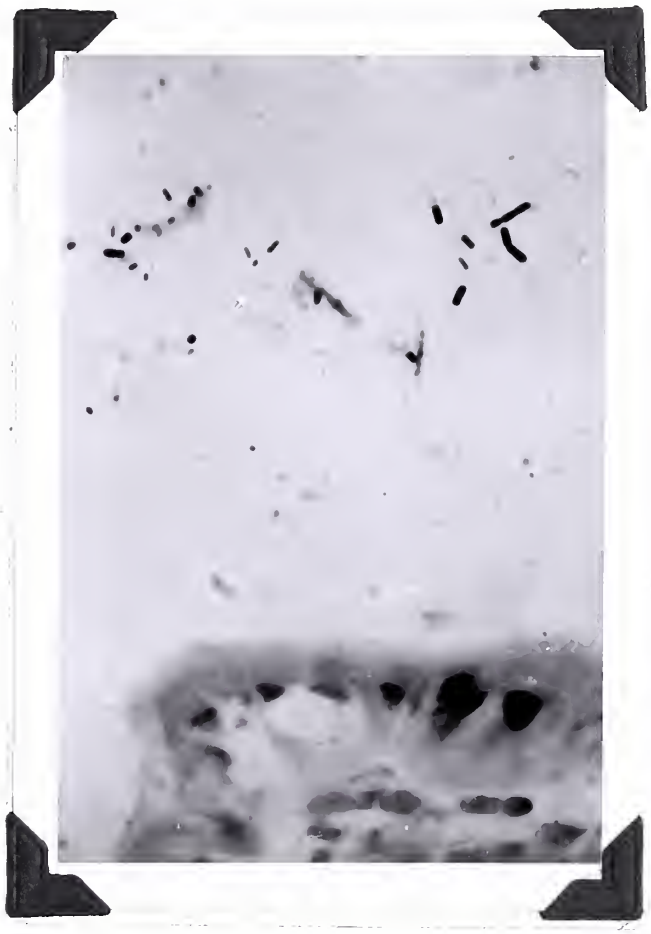


FIG. 6

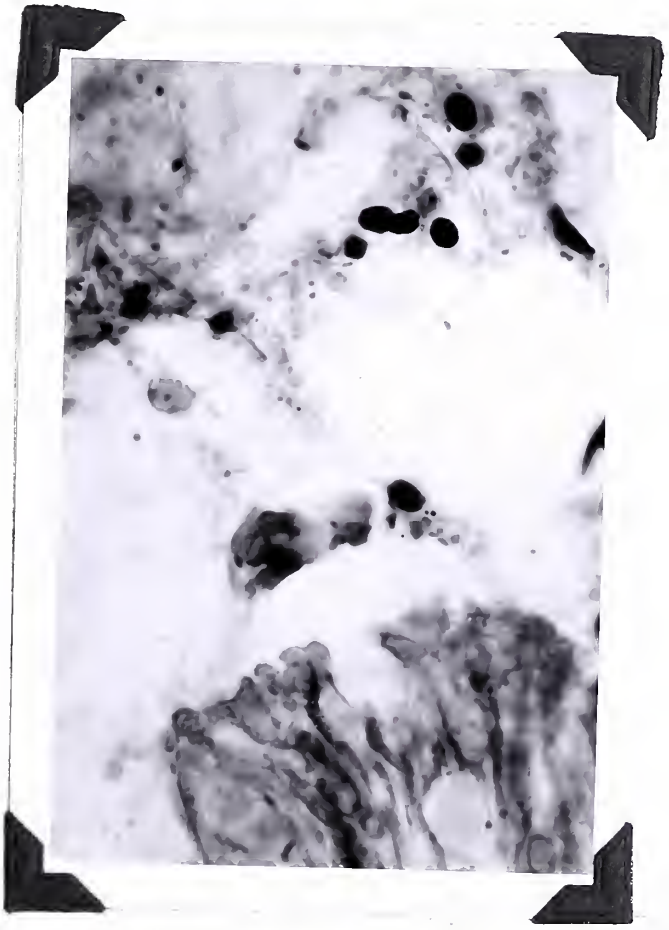


FIG. 7

Legends

FIG. 6

Oil immersion photomicrograph of the colonic bacteria from an unirradiated adult mouse. Gram-negative organisms (eg. white arrow) appear as gray rods; no fusiforms are seen. Gram-positive organisms are black. The superficial colonic epithelium is intact. (Reproduction ratio x960).

FIG. 7

Oil immersion photomicrograph of the colonic lumen from an antibiotic-fed adult mouse sacrificed ten days after irradiation. Note the striking absence of bacteria when compared to FIG. 6 and the presence of yeast forms. (Reproduction ratio x960).

Fig. 8

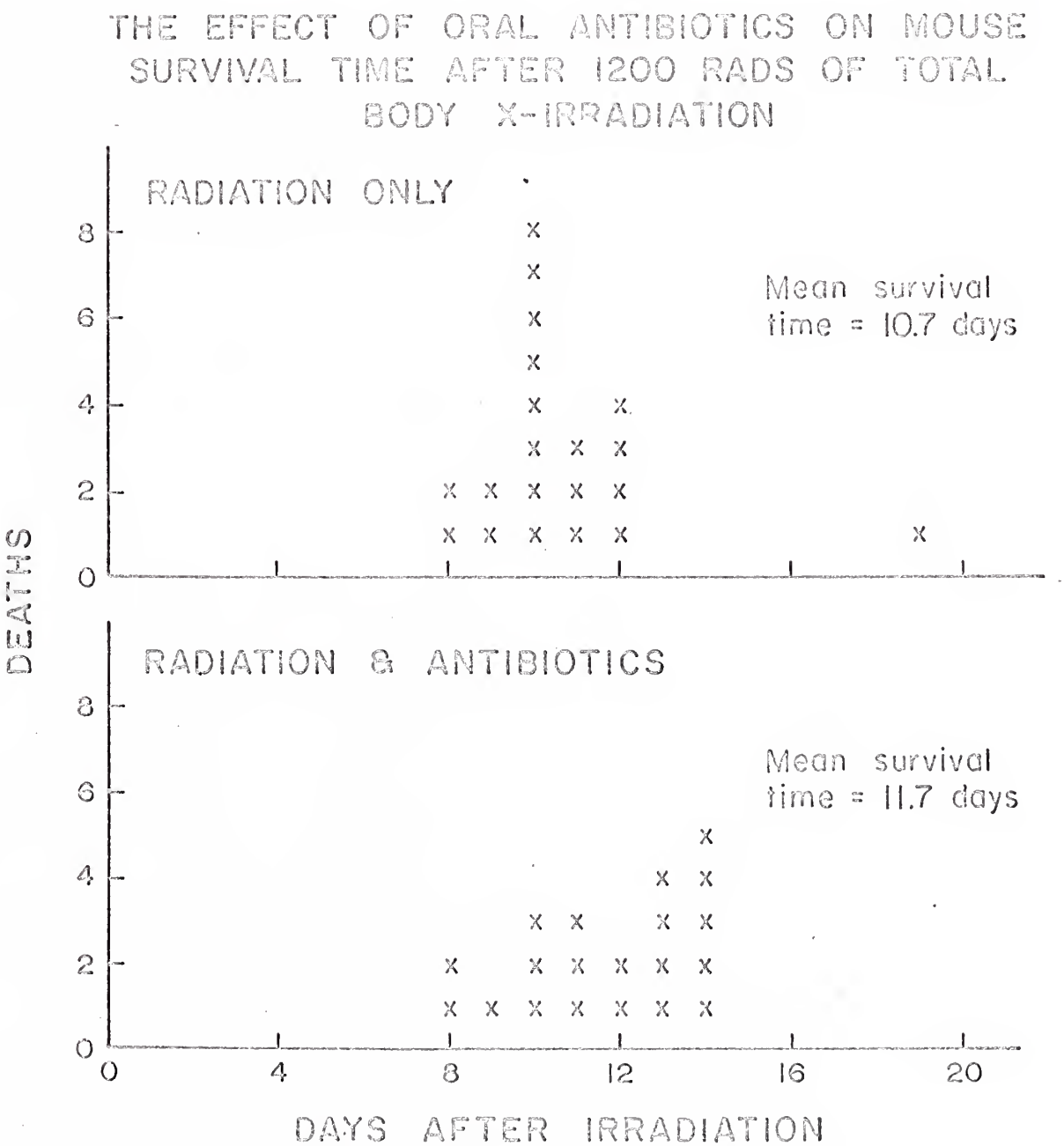
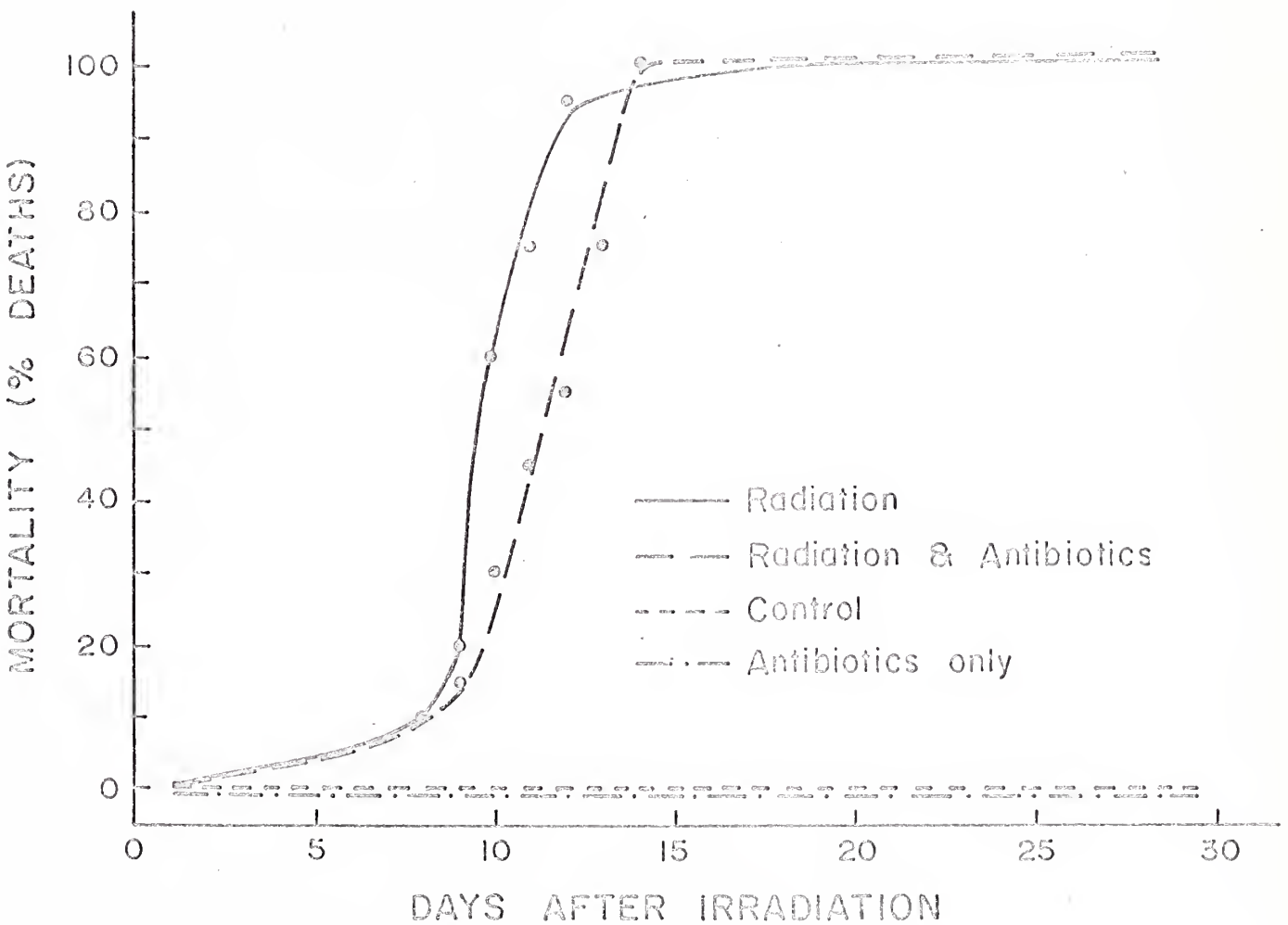

THE EFFECT OF ORAL ANTIBIOTICS ON MOUSE SURVIVAL TIME AFTER 1200 RADS OF TOTAL BODY X-IRRADIATION
RADIATION ONLY
Mean survival time = 10.7 days
RADIATION & ANTIBIOTICS
Mean survival time = 11.7 days
DEATHS
0
2
4
6
8
0
4
8
12
16
20
DAYS AFTER IRRADIATION

Fig. 9

THE EFFECT OF ORAL ANTIBIOTICS ON MORTALITY FOLLOWING 1200 RADS OF TOTAL BODY X-IRRADIATION



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